



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁷ : C12N 15/86, 15/87, A61K 48/00, 47/48, 38/17	A1	(11) International Publication Number: WO 00/29600 (43) International Publication Date: 25 May 2000 (25.05.00)
(21) International Application Number: PCT/US99/27365 (22) International Filing Date: 19 November 1999 (19.11.99) (30) Priority Data: 60/109,236 19 November 1998 (19.11.98) US 60/128,330 8 April 1999 (08.04.99) US (71) Applicants (for all designated States except US): GEORGE-TOWN UNIVERSITY [US/US]; 37th & O Streets, N.W., Washington, DC 20057 (US). SYNERGENE THERAPEUTICS, INC. [US/US]; 2022 R Street, N.W., Washington, DC 20009-1012 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): CHANG, Esther, H. [US/US]; 7508 Vale Street, Chevy Chase, MD 20815 (US). PIROLLO, Kathleen [US/US]; 2001 N. Adams Street #1031, Arlington, VA 22201 (US). XU, Liang [CN/US]; 1200 N. Queen Street #608, Arlington, VA 22209 (US). ALEXANDER, William [US/US]; 1211 Allison Drive, Rockville, MD 20851 (US). (74) Agents: SAXE, Stephen, A. et al.; Rothwell, Figg, Ernst & Kurz, Suite 701 East, Columbia Square, 555 13th Street N.W., Washington, DC 20004 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: SYSTEMIC VIRAL/LIGAND GENE DELIVERY SYSTEM AND GENE THERAPY (57) Abstract <p>The present invention relates to gene transfer and gene therapy technology. More specifically, the invention provides compositions and methods for targeted virus delivery. The method utilizes a method of mixing the virus, which may be a recombinant virus which will express a protein of interest or a nucleic acid of interest, with a cell-targeting ligand, e.g., transferrin. The virus and ligand are mixed without crosslinkers or agents which would covalently bond the virus and ligand. This simple mixing causes less inactivation than chemically linking the ligand to the virus and therefore results in a more active therapeutic composition than obtained by methods which utilize crosslinking agents.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

TITLE OF THE INVENTION

SYSTEMIC VIRAL/LIGAND GENE DELIVERY SYSTEM AND GENE THERAPY

BACKGROUND OF THE INVENTION

5 1. Technical Field

The present invention relates to improvements to gene transfer and gene therapy technology. More specifically, the invention provides compositions and methods for targeted *in vitro* and *in vivo* viral delivery of nucleic acids into human and other animals to a specific organ, tissue, or tumor. The use of this invention to deliver a therapeutic gene, e.g., wtp53, can result in increased sensitivity to conventional radiation and chemotherapies.

10 2. Description of the Background Art

Gene delivery and gene therapy using viral vectors have been the subject of considerable research. A long-standing goal in gene therapy for cancer is a systemic delivery system that selectively targets tumor cells, including metastases. Nucleic acids can be introduced into cells via viral vectors in order to produce a desired therapeutic effect upon those cells. For example, a gene can be introduced to replace a defective gene that interferes with cell function, e.g., p53. Nucleic acids may also be introduced into cells in order to produce a desired therapeutic effect in the host animal, e.g., for vaccination, immunotherapy, or anti-sense expression.

Viral vectors have been developed to take advantage of the cell entry mechanisms used by viruses to transfer their nucleic acids into host cells. Recombinant retroviral and adenoviral vectors have been constructed using this strategy to achieve gene transfer *in vitro* and *in vivo*. Approximately 80% of the gene therapy protocols that have been approved for clinical trial utilize viral vectors (Wivel and Wilson, 1998). Adenoviral vectors offer advantages for some forms of gene therapy because they can enter non-dividing cells and carry a relatively large (>8 kb) payload of foreign DNA (Berkner, 1988). Moreover, adenoviral particles can be purified and produced in titers greater than 10^{11} PFU/mL (Wivel and Wilson, 1998). One disadvantage of these systems, however, is the limited cell tropism of the viruses, and the significant problem of targeting viral particles has yet to be solved for any of the therapeutic viruses currently being used in clinical trials for cancer. Accordingly, methods to alter cell tropism have been developed and continue to be sought.

A system which changes the tropism of retroviruses by means of a bifunctional conjugate has been described (Roux et al., 1989). The bifunctional conjugate contains an antibody directed against the viral coat and, on the other end, an antibody directed to a specific cell membrane marker for the target cell.

5 Goud et al. (1988) described bifunctional conjugates which consist of two monoclonal antibodies (MAbs). The MAbs were directed against the gp70 coat protein of the Moloney retrovirus and the human transferrin receptor. These conjugates allowed the retrovirus to penetrate into the otherwise non-permissive target cells.

10 WO 92/06180 (Wu et al., 1992) describes a method for changing the tropism of a virus by providing the surface of a virus with a molecule that binds to a target cell surface receptor, producing a virus with a specificity for cells with the cell surface receptor. In the disclosure a retrovirus or hepatitis B virus is chemically modified with carbohydrate molecules which bind to the asialoglycoprotein receptor. Wu et al. disclose only *in vitro* methods for the introduction of foreign genes into cells.

15 Adenoviral vectors offer advantages for some forms of gene therapy because they can enter non-dividing cells and can carry a foreign DNA sequence of about 8 kb. Adenovirus particles can be purified and produced in titers greater than 10^{11} PFUs/mL.

20 One restriction on the use of recombinant adenoviruses is their limited ability to target specific cell types. A number of studies have reported gene transfer using non-recombinant adenoviruses with DNA complexes through receptor-mediated endocytosis, with the adenovirus providing the ability to release the contents of endosomes (Cotten et al., 1992; Wagner et al., 1992). These procedures use transferrin-polylysine/DNA complexes to internalize the bound or unbound adenoviruses. Wagner et al. (1992) modified an adenoviral vector by conjugating to polylysine and complexed this with conjugates of transferrin-polylysine/DNA, producing ternary
25 transferrin-polylysine/adenovirus-polylysine/DNA complexes. A similar approach to targeting is the linking of transferrin (Tf) to adenovirus particles to take advantage of the fact that the transferrin receptor (TfR) is elevated on many tumor types (Miyamoto et al., 1994; Baselga and Mendelsohn, 1994). Schwarzenberger et al. (1997) describe molecular conjugate vectors (MCVs). MCVs are constructed by condensing a plasmid containing the gene of interest with
30 polylysine (PL), PL linked to a replication-incompetent adenovirus (endosomolytic agent), and PL linked to streptavidin for targeting with biotinylated ligands. However, it has been reported

(Cotten et al., 1992; Wagner et al., 1992; Schwarzenberger et al., 1997) that current methods of covalent coupling of Tf to the adenovirus, or the generation of Tf-polylysine-adenovirus conjugates often results in decreased infectivity, possibly due to the harsh conditions required to produce the Tf-modified viruses.

5 Another approach entails crosslinking the Fab fragment of a neutralizing anti-fiber or anti-knob monoclonal antibody to a ligand, such as folate or FGF2 (Rogers et al., 1997; Douglas et al., 1996). Adenoviral vectors complexed with the chimeric Fab-ligand have shown some promise in tumor localization and exhibit reduced liver toxicity *in vivo* as compared to native adenovirus.

10 Curiel et al., (US Patent Nos. 5,521,291 and 5,547,932) disclose multiple adenoviral-polycation conjugates for internalizing nucleic acids into eukaryotic cells. Some of these conjugates use transferrin which is bound to a substance having an affinity for nucleic acid as an internalizing factor.

15 Cotten et al. (US Patent No. 5,693,509) disclose adenoviruses with the reported ability to penetrate efficiently into cells into which they cannot normally penetrate, while retaining their capacity for gene expression and/or their endosomolytic properties. Transferrin is covalently bound to adenovirus particles so that they can undergo receptor-mediated endocytosis. The method oxidizes transferrin into a form which contains aldehyde groups in the carbohydrate moiety and couples the oxidized transferrin to the adenovirus under reducing conditions. It is
20 also disclosed that it is unpredictable whether infectivity is maintained after modification.

25 Low et al. (US Patent Nos. 5,108,921; 5,416,016; and 5,635,382) disclose methods for enhancing transmembrane transport of exogenous molecules using ligands such as folate, biotin, thiamine, and their analogs. The methods may also employ anti-idiotypic antibodies or other molecules capable of binding to the ligand's receptors. Other ligands disclosed include niacin, pantothenic acid, riboflavin, pyridoxal, and ascorbic acid.

 Xu et al. (1997) and Xu et al. (1999) found that the addition of Tf to a cationic liposome was able to increase significantly the ability of the liposomes to deliver exogenous genes, including the normal p53 gene, both *in vitro* and *in vivo*. Most significantly, they achieved highly selective targeting to a wide variety of human tumor cells growing as xenografts in nude mice.

The publications and other materials used herein to illuminate the background of the invention or provide additional details respecting the practice, are incorporated by reference, and for convenience are respectively grouped in the appended List of References.

5 SUMMARY OF THE INVENTION

The present invention provides improvements to the administration of viral vectors for, for example, viral-mediated gene delivery to target cells.

10 In one aspect, the invention provides a composition comprising an admixture of a viral vector capable of delivering a nucleic acid or other therapeutic molecule of interest and a ligand capable of effecting or enhancing the binding or tropism of the viral vector to a target cell. The disclosed method of producing the admixture avoids inactivation of viral particles that can otherwise be caused by harsh chemical processing. The admixture is prepared in a simple manner and is suitable for systemic (e.g., parenteral) administration to a human patient.

15 In another aspect, the invention provides a method for administering the aforesaid admixture, systemically *in vivo* to a human or other animal, so as to accomplish targeted delivery of the contents of the viral particle.

In another aspect, the use of the invention to deliver a therapeutic gene, wild-type p53, will lead to sensitization to radiation and chemotherapeutic agents.

20 BRIEF DESCRIPTION OF THE FIGURES

25 Figure 1. β -galactosidase reporter gene expression in JSQ-3 cells after infection with Tf-targeted adenoviral vector carrying the lacZ gene. 5×10^4 JSQ-3 cells/well were plated in a 24-well plate. 24 hours later the cells were washed once with EMEM without serum and 0.3 mL EMEM without serum or antibiotics was added to each well. The Ad5LacZ or Tf-Ad5LacZ complexes at different ratios of transferrin to virus in 200 μ L EMEM were added to duplicate wells. Ratios of 5×10^2 to 5×10^5 Tf molecules/virion were used. The virus to cell ratios were 500 and 1000 viral particles/cell (pt/cell). After 4 hours incubation at 37°C, 5% CO₂, with mixing by rotating the test tube once every 2 minutes, 0.5 mL EMEM with 20% serum was added to the wells. After 2 days in culture, the cells were washed once with PBS, and lysed in 1X reporter lysis buffer (Promega). The cell lysates were treated with 100 μ L of 150 μ M O-nitrophenyl- β -galactopyranoside in 20 mM Tris (pH 7.5) containing 1 mM MgCl₂ and 450 μ M β -

30

mercaptoethanol at 37°C for 30 minutes. The reaction was stopped by the addition of 150 μ L/well of 1 M Na₂CO₃ and the absorbance was measured at 405 nm. Purified β -galactosidase was used to make a standard curve. The results were expressed as milliUnit (mU) of β -galactosidase equivalent per mg of total protein.

5 Figures 2A-F. Histochemical analysis of Tf-targeted adenoviral, systemic delivery of the β -galactosidase reporter gene in a mouse xenograft model. Athymic nude mice carrying DU145 xenograft tumors were i.v. injected one time with Tf-AdLacZ. Three days after injection, the animals were euthanized, and the tumor and normal tissues were excised and stained with X-gal. Figure 2A shows tumor from an animal systemically treated with Tf-Adp53 at a ratio of 1.5×10^5 Tf molecules/virion. Figure 2B shows liver corresponding to Figure 2A. Figure 2C shows tumor from an animal systemically treated with Tf-Adp53 at a ratio of 2.9×10^5 Tf molecules/virion. Figure 2D shows liver corresponding to Figure 2C. Figure 2E shows tumor from an animal systemically treated with Tf-Adp53 at a ratio of 5.8×10^5 Tf molecules/virion. Figure 2F shows liver corresponding to Figure 2E. Bar = 50 μ m.

15 Figure 3. Exogenous wtp53 expression in DU145 xenograft tumors after i.v. injection of Tf-Adp53. Athymic nude mice carrying DU145 xenograft tumors were i.v. injected with either Tf-Adp53 or untargeted Adp53. 48 hours later the animals were euthanized, the tumor and normal tissues excised, and protein isolated for Western blot analysis. The protein isolated from the tumor and organs of an untreated mouse, as well as the parental DU145 cells, were included as controls. 100 μ g of total protein of each of these samples was loaded/lane. 2.5 μ g total protein of DU145 cells infected *in vitro* with Adp53 was also included. The p53 protein bands were detected using the monoclonal anti-p53 antibody Ab-2 and the ECL Western blot kit. Band 1 = Exogenous human wtp53; Band 2 = Endogenous human DU145 p53; Band 3 = Endogenous mouse p53.

25 Figure 4. Effect of the combination of systemically delivered, tumor-targeted adenoviral-p53 and radiation treatment on JSQ-3 xenograft tumors *in vivo*. Tf-Adp53 was produced at a ratio of 1×10^5 Tf molecules/virion. 1×10^{10} viral particles/mouse/injection (equivalent to 3×10^8 pfu) of Tf-Adp53 or untargeted Adp53 were injected into the tail vein of athymic nude mice carrying JSQ-3 xenograft tumors of 100-200 mm³. Beginning the day after the first i.v. injection, 30 Gy of ionizing radiation was administered to the animals at the site of the tumor in 2 Gy daily fractionated doses. No tumor regrowth in the animals receiving the combination treatment was

30

observed 8 months after cessation of treatment. As the error was too small to be visualized, no error bars are present in the Tf-Adp53 (+) Radiation group. The bar represents the duration of treatment (approximately 3 weeks). All animal experiments were performed in accordance with Georgetown University Institutional Guidelines for the care and use of laboratory animals.

5 Figures 5A-H. Chemosensitization of B₁₆ mouse lung metastases to cisplatin (CDDP) by systemically delivered, tumor-targeted adenoviral-p53. The metastases were induced in normal, syngeneic C57/Bl/6 mice by the intravenous injection of 1×10^5 cells. Four days later treatment was begun. Tf-Adp53 and control Tf-AdLacZ were produced at the ratio of 1.5×10^5 Tf molecules/virion. 1×10^{10} viral particles/mouse/injection (equivalent to 3×10^8 pfu) of either
10 Adp53, Tf-Adp53 or Tf-AdLacZ were i.v. administered 3 times/week to a total of 12-13 doses. CDDP was intraperitoneally injected at 3-5mg/kg every 2-4 days for a total of 8-13 doses. The lungs were excised from the animals after one round of treatment. Lungs from animal treated with: No treatment (Figures 5A and 5E); CDDP alone (Figure 5B); untargeted Ad-p53 plus CDDP (Figure 5C); Tf-LacZ plus CDDP (Figure 5F); Tf-Adp53 alone (Figure 5G); Tf-Adp53
15 plus CDDP (Figures 5D and 5H).

Figure 6. Effect of the combination of systemically delivered, tumor-targeted adenoviral-p53 and chemotherapy on MDA-MB-435 xenograft tumors *in vivo*.

Figure 7. Expression of β -galactosidase in intratumorally injected DU145 cells which were injected with untargeted adenovirus with a LacZ or with transferrin targeted adenovirus
20 with LacZ.

DETAILED DESCRIPTION OF THE INVENTION

The normal development of mice lacking wtp53 and the observations of a post-irradiation G1 block in p53-expressing cells suggests that wtp53 functions in the regulation of the cell after
25 DNA damage or stress rather than during proliferation and development. Since it appears that many conventional anti-cancer therapies (chemotherapeutics and radiation) induce DNA damage and appear to work by inducing apoptosis, alterations in the p53 pathway could conceivably lead to failure of therapeutic regimens.

Lack of wtp53 function has also been associated with an increase in radiation resistance.
30 The presence of mtp53 and the consequent absence of a G1 block have also been found to correlate with increased radiation resistance in some human tumors and cell lines. These include

human tumor cell lines representative of head and neck, lymphoma, bladder, breast, thyroid, ovary and brain cancer.

Based on these considerations, gene therapy to restore wtp53 function in tumor cells should re-establish the p53-dependent cell cycle checkpoints and the apoptotic pathway thus leading to the reversal of the chemo-/radio-resistant phenotypes. Consistent with this model, chemosensitivity, along with apoptosis, was restored by expression of wtp53 in non-small cell lung carcinoma mouse xenografts carrying mtp53. Chemosensitivity of xenografts involving the p53-null lung tumor cell line H1299 and T98G glioblastoma cells and sensitivity of WiDr colon cancer xenografts to cisplatin has been demonstrated. Increased cell killing by doxorubicin or mitomycin C was also shown in SK-Br-3 breast tumor cells by adenoviral transduction of wtp53. However, some conflicting reports indicate that the relationship between p53 expression and chemoresistance may have a tissue or cell type-specific component. The transfection of wtp53 by an adenoviral vector has also been shown to sensitize ovarian and colo-rectal tumor cells to radiation. It has also been reported that adenoviral-mediated wtp53 delivery did restore functional apoptosis in a radiation-resistant squamous cell carcinoma of the head and neck (SCCHN) tumor line resulting in radiosensitization of these cells *in vitro*. More significantly, the combination of intratumorally injected adeno-wtp53 and radiation led to complete and long-term tumor regression of established SCCHN xenograft tumors.

The current invention departs from the conventional use of intratumoral injection of untargeted viral vectors or even systemic delivery of untargeted vectors, for the delivery of therapeutic molecules for gene therapy, for example as disclosed by Roth et al. (U.S. Patent No. 5, 747,469).

The data presented herein demonstrates the superior ability of such complexes to specifically target and sensitize tumor cells (due to expression of the wtp53 gene), both primary and metastatic tumors, to radiation and/or chemotherapy both *in vitro* and *in vivo*.

The present invention addresses the need to deliver therapeutic molecules systemically with a high degree of target cell specificity and high efficiency. When systemically administered, this delivery system is capable of reaching, and specifically targeting, metastatic as well as primary disease, when the target cells are human cancer cells. As a result of delivery of the normal, wild type version of the tumor suppressor gene p53 by means of this system, the inventors demonstrated that the tumors are sensitized to radiation therapy and/or chemotherapy.

The high transfection efficiency of this system results in such a high degree of sensitization that not only is there growth inhibition of the cancer but pre-existing tumors and metastases are completely eliminated for an extended period of time.

Specific embodiments of the invention provide pharmaceutically acceptable compositions comprising an admixture of transferrin ligand and viral vector particles capable of delivering a nucleic acid to target host cells. Simply admixing viral vectors (e.g., retroviral or adenoviral vectors) with a cell-targeting ligand in a suitable vehicle such as sterile-water-for-injection increases transfection efficiency over that obtained with the viral vector alone. A simple admixture of viral vector and transferrin, for example, increases the transfection of cells, e.g., human cancer cells, expressing the transferrin receptor.

The use of transferrin is especially advantageous in connection with gene transfer into, or gene therapy for, a wide variety of human cancers. A wide variety of human cancer cells contain transferrin receptors. The presence of transferrin in the admixtures of the present invention permits the viral vectors to efficiently and specifically target those cancer cells.

Other ligands, such as proteins, peptides, hormones, antibodies and antibody fragments will be useful for specifically targeting the viral vectors to cells containing receptors for such ligands or which can internalize the ligand by receptor-mediated endocytosis. The ligand, for example, can be a native or recombinant protein that functions to enhance the binding of the viral vector to a target cell. Examples of ligands include insulin, toxins, EGF, VEGF, FGF, IGF, heregulin, other viral or bacterial proteins, estrogen and progesterone.

While the invention encompasses the use of a cell-targeting ligand which occurs naturally on one type of virus when this ligand is mixed with a second type of virus, the invention does not encompass the use of a cell-targeting ligand in its naturally occurring association with the virus which encoded it. The invention does encompass the mixture of a cell-targeting ligand encoded by a virus in association with the virus type which encodes said ligand when the ligand is present in an amount higher than normally found in association with the naturally occurring virus.

The method by which a complex is formed between the ligand and the viral particle is such that a large number of ligand molecules coat the surface of the viral particle and increase the stability thereof as it travels through the blood stream. Moreover, the high number of ligand molecules on the surface may also serve to decrease the immunogenicity of the virus by blocking viral antigens.

The invention includes the use of recombinant expression viruses. Viral vectors, such as recombinant adenovirus, AAV vectors (U.S. Pat. No. 5,139,941), retroviral vectors, herpes simplex virus (U.S. Pat. No. 5,288,641), cytomegalovirus (CMV), vaccinia virus, fowlpoxvirus (FPV), canarypoxvirus (CPV)(U.S. Pat. Nos. 5,833,975; 5,762,938; and 5,378,457), Sindbis virus, chimeric or hybrid viruses and the like may be used in accordance with the invention. Replication-competent or oncolytic viruses also can be used in accordance with the invention.

The invention also provides methods for preparing a viral vector-transferrin admixture which advantageously avoids the harsh chemicals and complicated processing steps that have been described in connection with previously used methods using MAbs, linkers, polylysine, etc., to link transferrin to viral vectors.

In accordance with the invention, ligand-viral admixtures can be prepared in any carrier or vehicle (typically an aqueous carrier) so as to provide a composition that is pharmaceutically suitable for *in vitro* or *in vivo* administration. The composition typically will be buffered to a suitable pH and can contain suitable auxiliary components such as osmolarity adjusting agents, antibiotics, etc.

The amounts of viral particles used in the admixtures and ultimately administered to the host animal (or administered to cells *in vitro*) will be determined by those skilled in this field based upon well-known principles of gene transfer and gene therapy described in the scientific literature. The admixtures of the invention are administered via methods analogous to those previously described for the administration of viral vectors so as to carry out *in vitro* or *in vivo* gene transfer or gene therapy. The invention improves upon existing technology by providing compositions and methods for the systemic administration of viral vectors. Parenteral administration (especially intravenous or intra-arterial administration) of the admixtures is preferred. It is anticipated that, in some cases, substantially (for example 30-fold) lower doses of viral particles can be administered due to the improved efficiency brought about by the invention. Alternatively, in other cases, known doses can be used, resulting in increased genetic transfer. Concentrations of viral particles, ligand and auxiliary agents within the compositions of the invention also will be suitably selected.

Specific embodiments of the invention provide for using the compositions of the invention in conjunction with radiation treatment and/or chemotherapy.

The invention is not limited to any particular viral vector, or to any particular route or mode of administration of the ligand-viral vector admixture compositions. The desired total dose can be determined experimentally, and can be provided to a patient in need of gene therapy in a single or in multiple administration(s).

5 The data presented in the Examples indicate that Tf-adenovirus complexes are capable of producing markedly higher levels of gene expression in tumors than that seen with untargeted adenoviral vectors. The gene delivery method described here is based upon the relatively simple method of producing the Tf-targeted viruses. The coupling is non-covalent and does not involve chemical reactions capable of producing unwanted and perhaps toxic side products and avoids
10 the harsh chemical conjugation and complicated processing steps that have been described in connection with previously used methods using MAbs, linkers, polylysine, etc., to link Tf to viral vectors. These chemical modifications of viruses inevitably lower the infectivity of the virus and can produce aggregates possibly too large to penetrate the tumor capillaries. While intratumorally injected viral gene therapy vectors including oncolytic viruses are in clinical trials,
15 no targeted viruses without covalent modifications are currently under clinical study.

 While it is becoming evident that single agent p53 gene therapy is not sufficient to completely eliminate tumors long term, the presently-described combination of Tf-targeted adenovirus and conventional radiation/chemotherapy was able to achieve not only growth inhibition, but tumor regression, demonstrating a synergistic effect.

20 The *in vivo* studies described herein demonstrate that the combination of systemic Tf-Adp53 gene therapy and conventional radiotherapy and/or chemotherapy is markedly more effective than either treatment alone. In the clinical setting, radiation doses of 65 to 75 Gy for gross tumor and 45 to 50 Gy for microscopic disease are commonly employed in the treatment of head and neck cancer. Given the known, adverse side effects associated with high doses of
25 radiation or chemotherapy, sensitization of tumors so as to permit a lowered effective dose of the conventional treatment would be of immense clinical benefit. Furthermore, in the case of radiation, systemic restoration of wtp53 function, resulting in a decrease in the radiation treatment dose found to be effective, would permit further therapeutic intervention for tumors which did reoccur.

30 The sensitization of tumors to chemotherapy and radiation will result in increased efficacy of current treatment modalities. Moreover, the potential also exists for this tumor specific

combination treatment to lower the necessary dose of both types of conventional anticancer modalities thereby lessening the severe side effects often associated with these treatments. In the *in vivo* studies described in the Examples, systemic administration of Tf-Adp53 in combination with radiation resulted in total and long-term tumor regression using as little as 3×10^8 pfu of the tumor-targeting virus. This dose is approximately equivalent to that employed by Kataoka et al. (1998) using untargeted Adp53 and 2-Me. In that study, partial tumor growth inhibition (two thirds reduction in lung colony count) was observed.

Although this system may well eliminate the need for intratumoral injection of gene therapy vectors, in the case of very aggressive cancers it may also be beneficial to use both systemic and intratumoral treatments. This system may also be adapted to assist in the delivery of other viral cancer treatments. For example, current trials of Onyx's oncolytic viruses do not support systemic delivery. ONYX-015 is a genetically modified adenovirus that efficiently replicates in and kills tumor cells deficient in wtp53 tumor suppressor activity ("p53-deficient" cells) and not in normal cells. The specific modification of the virus prevents it from replicating efficiently in normal cells. Clinical studies with ONYX-015 are currently underway for head and neck cancer, pancreatic cancer and ovarian carcinoma (Heise et al., 1997; Hall et al., 1998; Linke, 1998; Kirn et al., 1998). Our ability to target viruses to tumors may significantly improve the efficacy of other virally based cancer treatments such as ONYX-015.

Most significantly, systemic administration means that both the primary tumor and distant metastases can be reached with therapeutic genes. This is in stark contrast to that which can be achieved with intratumoral injection. The method described here is adaptable to targeting any existing recombinant viruses. It is also independent of the gene to be delivered. Additionally, this system could, as mentioned above, also be used with oncolytic viruses. The use of Tf for targeting is especially attractive in connection with p53 gene therapy for human cancers in that a broad spectrum of cancers express elevated levels of the TfR, and in over 50% of cancers, the p53 gene has been implicated. Therefore, these findings demonstrate the clinical potential of this Tf-targeted adenoviral delivery system as a new, more efficient and effective form of gene therapy for cancer, one which will help to fulfill the initial promise of gene therapy in the war against this disease.

Specific, illustrative embodiments of the present invention are provided in the following Examples.

Example 1Transferrin enhances adenoviral transduction efficiency

A. Preparation of Transferrin-Adenovirus Admixture

Holo-transferrin (Tf, iron-saturated, Sigma) was dissolved in sterile water at 5 mg/mL. Replication deficient adenovirus serotype 5, designated Ad5LacZ (Ad5CMVntbeta-gal, Gene Transfer Vector Core, University of Iowa), containing the *E. coli* LacZ gene under control of the CMV promoter, at a concentration of 1.1×10^{12} particles(pt)/mL (which contained 5.5×10^9 plaque forming units, pfu/mL) in PBS plus 3% sucrose, was used in the study. Tf was first diluted to 0.5 mg/mL in 10 mM HEPES buffer, pH 7.4, then the Tf was added to 50 μ L HEPES buffer in a 10-fold serial dilution. Ad5LacZ was then added to the tubes so that the Tf to virus ratios ranged from 1×10^2 up to 1×10^6 Tf molecules/virion. The tubes were incubated at room temperature for 10-15 minutes, with rocking (rotating the tubes once every two minutes), and then 150 μ L EMEM without serum was added to each tube.

B. *In vitro* transduction using adenovirus/Tf admixture

We have employed a replication deficient adenovirus of serotype 5 termed AdLacZ (containing the *E. coli* LacZ gene under control of the CMV promoter) and the Tf-modified form of this virus (Tf-AdLacZ). To optimize the ability of Tf-AdLacZ to deliver the reporter gene, cultures of the cell line JSQ-3 (Weichselbaum et al., 1988), derived from a human squamous cell carcinoma of the head and neck (SCCHN), were infected (Bischoff et al., 1996) with AdLacZ or with Tf-AdLacZ produced using different ratios of Tf to virus and virus particles to cell.

5×10^4 JSQ-3 cells/well were plated in a 24-well plate. 24 hours later the cells were washed once with EMEM without serum, 0.3 mL EMEM without serum or antibiotics was added to each well. The Ad5LacZ or Tf-Ad5LacZ complexes at different ratios of transferrin to virus in 200 μ L EMEM were added to duplicate wells. The virus to cell ratio ranged from 20 up to 2000 viral particles/cell (pt/cell). After 4 hours incubation at 37°C, 5% CO₂, with occasional rocking, 0.5 mL EMEM with 20% serum was added to the wells. After 2 days in culture, the cells were washed once with PBS, and lysed in 1X reporter lysis buffer (Promega). The cell lysates were treated with 100 μ L of 150 μ M O-nitrophenyl- β -galactopyranoside in 20 mM Tris (pH 7.5) containing 1 mM MgCl₂ and 450 μ M β -mercaptoethanol at 37°C for 30 minutes. The reaction was stopped by the addition of 150 μ L/well of 1 M Na₂CO₃. The absorbance was

measured at 405 nm. Purified β -galactosidase (Boehringer) was used to make a standard curve. The results were expressed as milliUnits (mU) of β -galactosidase equivalent per mg of total protein.

5 C. Histochemical staining

For histochemical studies of Tf-Ad5LacZ transduction, 60% confluent cells in 24-well plates were transfected for 5 hours with transfection solutions as described above. After an additional 2 days in culture, the cells were fixed and stained with X-gal (Xu et al., 1997). Transfection efficiency was calculated as the percentage of blue-stained cells.

10

D. Results and Discussion

At certain ratios of Tf to virus or of virus to cell, expression with the Tf-AdLacZ was between 3- to 4-fold higher than that seen with the untargeted AdLacZ (see Figure 1). At a viral dose of 500 pt/cell or 2.5 MOI (multiplicity of infection, or pfu/cell), 10 mU/mg protein of reporter gene product β -galactosidase was expressed by Ad5LacZ alone. Transduction with the transferrin-virus admixture Tf-Ad5LacZ (500 Tf molecules/pt) produced 25 mU/mg protein of reporter gene expression, Tf-Ad5LacZ (5,000 Tf molecules/pt) produced 30 mU/mg expression, and Tf-Ad5LacZ (50,000 Tf molecules/pt) produced 38.8 mU/mg expression, which represents 2.5, 3, and 3.8-fold, respectively, more gene transduction than attained with Ad5LacZ alone. At a dose of 1,000 pt/cell or 5 MOI, Tf-Ad5LacZ (500 Tf molecules/pt) gave 2.4-fold more reporter gene expression and Tf-Ad5LacZ (5000 Tf molecules/pt) gave 3.3-fold more gene expression than Ad5LacZ only. Tf-Ad5LacZ (50,000 Tf molecules/pt) gave 2.6-fold more expression, seeming to reach saturation. Therefore, the optimal ratio of Tf-Ad5LacZ complex appeared to be about 500-50,000 Tf molecules/pt, preferably about 5000 Tf molecules/pt. It is thought that the large number of transferrin molecules used to coat the surface of the viral particles increased its stability as it traveled through the bloodstream. The large number of transferrin molecules on the virion surface can also serve to decrease the immunogenicity of the virus by blocking viral antigen exposure.

25

Histochemical staining showed that Ad5LacZ alone gave 20-30% transduction efficiency while transferrin complexed adenovirus Tf-Ad5LacZ (5000 Tf molecules/pt) gave 70-90% efficiency.

30

The above results demonstrated that adenoviral-transferrin admixtures can substantially enhance adenoviral gene transduction.

Example 2

5

Transferrin-targeted Systemic Adenoviral Gene Delivery in Nude Mouse JSQ-3 Xenograft Model

A. Preparation of transferrin-adenovirus complex

The transferrin-Ad5LacZ complex was prepared by means similar to those used for the preparation described in Example 1. 1×10^9 - 1×10^{10} pt Ad5LacZ (1×10^{12} pt/mL in PBS plus 3% sucrose) was mixed with different amounts of Tf (4 to 5 mg/mL in water) at ratios ranging from 1 μ g to 1 mg Tf/ 1×10^{10} pt, or 7.5×10^2 - 7.5×10^5 Tf molecules/virion. The mixtures were incubated at room temperature for 5-10 minutes with rocking (rotation of the tubes once every two minutes) to permit the Tf-Ad5LacZ complex to form. PBS (pH 7.4) was added to each tube to dilute to 1×10^9 - 1×10^{10} pt/0.2-0.3 mL/mouse injection.

15

Two types of human tumors were established as xenografts in nude mice by subcutaneous injection of either the SCCHN cell line used in the culture experiments above (JSQ-3) or the human prostate cancer cell line DU145 (Isaacs et al., 1991; Asgari et al., 1997). The nude mouse tumor model was established by subcutaneous injection of JSQ-3 cells or DU145 cells into the flank of 4-6 week old female nude mice (Xu et al., 1997). The tumors were allowed to grow to a size of 1-2 cm³. 1×10^9 - 1×10^{10} pt Ad5LacZ complexed with different amounts of Tf in 200-300 μ L were injected into each mouse via the tail vein with a 1 cc syringe and a 30 G needle. In the control group, Ad5LacZ was injected. Three days after injection, the tumors as well as mouse organs were excised, cut into 1 mm sections, washed once with PBS, and fixed with 2% formaldehyde/0.2% glutaraldehyde for 4 hours at room temperature. The fixed tumor sections were washed 4 times, each for 1 hour, and stained with X-Gal solution plus 0.1% NP-40 (pH 8.5) at 37°C overnight. The stained tumor sections were embedded and sectioned using normal histological procedures and counter-stained with nuclear fast red. Four sections per tumor were examined to evaluate the β -galactosidase gene expression, as indicated by the blue stained cells.

20

25

Another tumor section was used for quantitative β -galactosidase assay. The tissues were homogenized and lysed in 1X reporter lysis buffer (Promega). The lysates were added to a 96-well plate and a quantitative β -galactosidase assay was carried out as described in Example 1.

30

In some experiments, the quantitative β -galactosidase assay was also performed using a Luminescent β -galactosidase Detection Kit II (Clontech).

B. Results and Discussion

5 To test systemic, targeted viral gene delivery, viral vectors were injected i.v. in well-established solid tumor models. Two solid tumor xenograft models were used to test the targeted viral gene delivery system. 1×10^{10} pt Ad5LacZ alone or complexed with different amounts of Tf were i.v. injected into nude mice bearing 1-2 cm³ size human tumor xenografts of JSQ-3 and DU145 cells. Three days later, the tumors injected with Tf-Ad5LacZ showed increased
10 X-Gal-stained blue cells, as compared with Ad5LacZ alone (<1%). The efficiency increased as a result of increased Tf/pt ratios, from 5% up to >35% (0.01 mg-0.6 mg/ 10^{10} pt). The efficiency started to decrease with Tf/pt ratios >0.6-1 mg/ 10^{10} pt (about 4.5 to 6×10^5 Tf molecules per virion). This is shown in Figures 2A, 2C, and 2E where the percent of β -galactosidase expressing cells (as indicated by X-gal staining) actually decreases as the ratio of Tf-
15 molecules/virion increases from 2.9×10^5 Tf/virion to 5.8×10^5 Tf/virion (Figures 2C and 2E). Moreover, at the ratio of 1.5×10^5 Tf/virion no β -galactosidase expression was evident in the liver (Figure 2B) or other organs including spleen and lung, while there was minimal, but clearly detectable, β -galactosidase expression in the liver at the ratio of 2.9×10^5 Tf molecules/virion (Figure 2D). In contrast, injection of Tf-AdLacZ produced with higher ratios of Tf/virion
20 resulted in notable liver staining (Figure 2F). Therefore, based upon these findings, the ratio of 1.5×10^5 Tf molecules/virion, which demonstrated significant tumor transfection efficiency, while maintaining the highest degree of tumor specificity was determined to be optimal for the studies. The optimal conditions appeared to be about 0.01-0.2 mg/ 10^{10} pt (7.5×10^3 - 1.5×10^5 Tf molecules per virion) for JSQ-3 and about 0.03-0.5 mg/ 10^{10} pt for DU145. Therefore, Tf/pt
25 ratios can be optimized *in vivo* in different tumor models. It should be noted that *in vitro* optimal Tf/pt ratios are much smaller than that of *in vivo*, such that more transferrin may be needed *in vivo* to stabilize the virus for improved targeting. The 0.2 mg Tf/ 10^{10} pt (1.5×10^5 Tf molecules per virion) ratio was used in subsequent *in vivo* gene therapy experiments for JSQ-3 tumors.

30 Quantitative β -galactosidase assays also confirmed the substantial increase of gene expression in tumors of mice i.v. injected with transferrin-targeted adenovirus, compared with that of adenovirus alone.

Targeted organ delivery of virus was also observed in livers of mice with i.v. injected Tf-Ad5LacZ complex, but liver delivery has a different preferred Tf/pt ratio, e.g. about 0.5 mg-1.3 mg Tf/ 10^{10} pt (3.75×10^5 - 9.75×10^5 Tf molecules per virion). In Ad5LacZ alone i.v.-injected mice, only a limited number of hepatocytes stained blue (<1-5%), while mice i.v.-injected with Tf-Ad5LacZ showed increased blue hepatocytes (10%-40%). The difference of preferred Tf/pt ratios between tumor-targeting and liver-targeting illustrates that systemic viral delivery systems according to the present invention can be optimized so as to be selective for different targets.

Example 3

Transferrin-targeted Adenoviral-Mediated Gene Delivery and Protein Expression of p53 *In Vivo* in a DU145 Xenograft Nude Mouse Model

The ability of the transferrin-targeted adenoviral vector to deliver the p53 gene selectively to tumors was examined. The replication deficient adenovirus serotype 5, carrying the normal human p53 gene was used in these studies. This virus, termed Adp53, was used to produce Tf-Adp53. Tf-Adp53 was produced by mixing Holo-Transferrin with Adp53 in 10 mM HEPES, pH 7.4, at a ratio of 1.5×10^5 Tf molecules/virion. After incubation for 10 minutes at 4°C, phosphate buffered saline (PBS), pH 7.4, was added to bring the final volume to 300 µL/mouse and made to a final concentration of 5% dextrose. Three days after i.v. injection of these viruses into nude mice bearing subcutaneous DU145 tumors, the mice were euthanized, the tumors and organs excised, and Western blot analysis for p53 protein expression performed (see Figure 3). The antibody used in these studies reacts with both normal and mutated forms of human p53 and cross-reacts with mouse p53. The p53 band representing the virally transduced wild-type p53 migrates in the gel above the mutated form of p53 found in the DU145 cells. This can be seen in the left two lanes of Figure 3 where DU145 cells are compared with DU145 cells infected in culture with Adp53. An upper band, representing the virally encoded wtp53, was evident in DU145 cells infected *in vitro* with Adp53. It should be noted that the first lane (DU145 + Adp53) contains only 2.5 µg of protein whereas all other lanes of Figure 3 contain 100 µg of total protein.

Western blot analysis of tumors from mice receiving the targeted Adp53 (i.e., Tf-Adp53) revealed an upper band (exogenous p53) and a lower band (endogenous DU145 p53) that merged

into what appears as a single large band. It is clear that there is significantly more exogenous p53 in tumors from mice receiving Tf-Adp53 than the tumors from the mice treated with the untargeted Adp53. Liver and other vital organs from the mouse treated with the targeted Tf-Adp53 displayed little or no exogenous wtp53. In contrast, treatment with untargeted Adp53, resulted in a higher level of exogenous p53 in the liver. As would be expected, tumors of untreated mice contained only the endogenous DU145 p53 and organs from these animals contained only endogenous mouse p53. These results further confirm that the Tf-Adp53, and not the untargeted Adp53, can selectively target tumors *in vivo*, and that p53 is efficiently expressed in the tumor tissue following i.v. administration.

Example 4

Transferrin-Targeted Systemic Adenoviral-Mediated Gene Delivery *In Vivo* in an SCCHN Xenograft Nude Mouse Model in Conjunction with Radiation Treatment

The ultimate test of the usefulness of a targeted adenoviral delivery system in gene therapy is its effectiveness in treating tumors when systemically administered. It has been established that loss of functional p53 can contribute to the radiation-resistant phenotype (Bristow et al., 1996). We previously demonstrated that the combination of wtp53 and conventional radiation treatment was able to eliminate established xenograft tumors long term (Xu et al., 1999; Pirollo et al., 1997). The results in this example show the ability of Tf-Adp53 to sensitize xenografts of human tumor cells to radiation therapy.

Xenografts were induced in 4-6 week old female athymic nude (NCR nu-nu) mice by the subcutaneous injection of 4×10^6 JSQ-3 cells (in MatrigelTM, a collagen matrix) on the lower back above the tail of each animal. The JSQ-3 cell line was derived from a recurrent SCCHN and is known to be highly radioresistant. Tumors were allowed to develop to a size of 100-200 mm³. The targeted adenovirus, designated Targeted Ad-p53, was prepared by mixing transferrin with adenovirus carrying DNA encoding wt p53 as described in Example 1. Adenovirus particles (pt) per plaque forming unit (pfu) was calculated for this experiment. The animals were divided into four groups: (i) Untreated (-) Radiation; (ii) Untargeted Ad-p53 (+) Radiation; (iii) Targeted Ad-p53 (-) Radiation; (iv) Targeted Ad-p53 (+) Radiation. The mice (in groups ii, iii and iv) were i.v. injected, via the tail vein, every three to four days with 1×10^{10} pt/mouse/injection (equivalent to approximately 3×10^8 pfu) of either targeted (an admixture of

Tf ligand and virus was injected) or untargeted (no ligand) Ad-p53. A total of 6 injections were administered. The day after the initial i.v. injection, the animals (in groups ii and iv) were secured in a lead holder, which permitted only the tumor area to be irradiated, and the first fractionated dose of 2.0 Gy of ^{137}Cs ionizing radiation administered using a J.L. Shepard and Associates Mark I irradiator. Thereafter, the animals were given 2.0 Gy/day for 5 consecutive days, followed by 2 days without radiation treatment. The cycle was repeated until a total of 30 Gy had been administered. The tumor sizes were measured weekly in a blinded manner.

The untreated animals and those receiving Targeted Ad-p53 without radiation were euthanized due to tumor burden by day 52 (see Figure 4). Treatment with Untargeted Ad-p53 plus radiation delayed tumor growth during the course of treatment. However, once treatment ceased, the tumors in these animals began to increase in size such that by day 121 they also had to be euthanized due to tumor burden. In contrast, the tumors in the animals receiving Tf-Targeted Ad-p53 in combination with radiation regressed completely, during and even after cessation of treatment, such that more than 8 months post treatment there was no recurrence of the tumors in these animals.

These results together with those in Figure 3 demonstrate that systemically administered Tf-targeted adenovirus can deliver wt-p53 selectively to tumors resulting in their sensitization to conventional radiotherapy. Most importantly, the combinatorial treatment of Tf-Adp53 plus radiation resulted in eradication of tumors long-term. Recently, Kataoka et al. (1998) reported that the combination of 2-methoxyestradiol (2-Me) and systemic delivery of untargeted Adp53 in a mouse model using A549 cells partially inhibits metastatic lung tumor growth. This combination treatment resulted in a two-thirds reduction in lung colony count. While certainly promising, the tumor cells remaining after this treatment would most certainly grow and eventually kill the animal. In contrast, our results with Tf-targeted Adp53 produced apparently total, long-term (currently 8 months out) regression of subcutaneous SCCHN tumors.

Example 5

Transferrin-Targeted Systemic Adenoviral-Mediated Gene Delivery *In Vivo* in an SCCHN Xenograft Nude Mouse Model in Conjunction with Radiation Treatment

JSQ-3 xenografts were induced in NCr nu-nu mice as in Example 4. Tumors were allowed to develop to a size of 50-60 mm³. The targeted adenovirus, with (Targeted Ad-p53) or

without (Targeted Ad) DNA encoding human wt p53, was prepared by mixing transferrin with adenovirus as described in Example 1. Adenovirus particles (pt) per plaque forming unit (pfu) was calculated. The animals were divided into five groups: (i) Untreated (-) Radiation; (ii) Untargeted Ad-p53 (+) Radiation; (iii) Targeted Ad (+) Radiation; (iv) Targeted Ad-p53 (-) Radiation; (v) Targeted Ad-p53 (+) Radiation. Mice were i.v. injected via the tail vein every three to four days with 3×10^9 pt/mouse/injection. A total of 5 injections were administered. Three days after the initial i.v. injection, the animals were secured in a lead holder, which permitted only the tumor area to be irradiated, and the first fractionated dose of 2.0 Gy of ^{137}Cs ionizing radiation was administered using a J.L. Shepard and Associates Mark I irradiator. Thereafter, the animals were given 2.0 Gy/day for 5 consecutive days, followed by 2 days without radiation treatment. The cycle was repeated until a total of 26 Gy had been administered. The tumor sizes were measured weekly in a blinded manner. As in Example 4, the tumors in the untreated animals and those receiving Targeted Ad-p53 without radiation demonstrated continuous growth such that by day 50 the animals were euthanized due to tumor burden. Tumors in the group treated with radiation and Targeted-Ad without wt p53 demonstrated some minimal radiation inhibition of growth during treatment, but tumors increased in volume once the treatment was ended. A similar but even more dramatic regrowth occurred post-treatment in the group of animals that received the Targeted-Ad-p53 but no radiation. This is in sharp contrast to those mice receiving the Targeted Ad-p53 in combination with radiation. As observed in Example 4, tumor regression continued in these animals more than eight months after the end of all treatment. Eight months in the lifespan of a mouse is equivalent to 30 years in a human life span.

Example 6

Transferrin-targeted Retroviral Gene Transduction

Retroviral vectors are one of the most widely used gene therapy vectors in clinical trials. As with adenoviral vectors, retroviral vectors exhibit poor specificity and significant immunogenicity.

Replication-deficient retrovirus containing the *E. coli* LacZ gene, RvLacZ (A Lac Z, Gene Transfer Vector Core, University of Iowa), at 1×10^{10} particles (pt)/mL containing 3×10^7 transforming unit (TU)/mL, was employed in this study. Transferrin and Tf-RvLacZ complex

was prepared similarly to that described in Example 1. Briefly, Tf was first diluted to 0.5 mg/mL in 10 mM HEPES buffer, pH 7.4, then different amounts of Tf were added to 50 μ L HEPES buffer in a serial dilution. RvLacZ was then added to the tubes so that the Tf to virus ratios ranged from 1×10^2 up to 1×10^6 Tf molecules/virion. The tubes were incubated at room temperature for 10-15 minutes with rocking every two minutes then 150 μ L of EMEM without serum was added to each tube. *In vitro* retroviral transduction was performed as described in Example 1. The virus to cell ratio ranged from 100 to 2000 viral pt/cell.

At a viral dose of 500 pt/cell or 1.5 MOI (or TU/cell), 3.4 mU/mg protein of β -galactosidase was expressed by the retrovirus RvLacZ alone. With transferrin-complexed virus, administration of Tf-RvLacZ (500 Tf molecules/pt) gave 6.8 mU/mg protein of β -galactosidase expression, while administration of Tf-RvLacZ (5000 Tf molecules/pt) gave 9 mU/mg expression, which represents 2- and 3-fold higher gene transduction than produced via the administration of RvLacZ alone. The increase of gene transduction plateaued at a ratio of 50000 Tf molecules/pt. At a dose of 1000 pt/cell or 3 MOI, Tf-RvLacZ (5000 Tf molecules/pt) produced 2.1-fold more reporter gene expression and Tf-RvLacZ (50000 Tf molecules/pt) produced 3-fold more expression than RvLacZ alone. Histochemical staining showed that RvLacZ alone had a 20-30% transduction efficiency while transferrin-complexed retrovirus Tf-RvLacZ (5000 Tf molecules/pt) had a 60-80% transduction efficiency. The results demonstrated that the administration of an admixture of transferrin and retrovirus can substantially enhance retroviral gene transduction.

Example 7

Transferrin-Targeted Systemic Adenoviral-Mediated Gene Delivery

In Vivo in a Syngeneic Mouse Model in Conjunction with Chemotherapy

The ability of the ligand-targeted, viral p53 delivery system to sensitize tumor cells to chemotherapy in an immune competent animal model was examined. The model chosen for these studies was the B₁₆ mouse melanoma lung metastases model. In multiple experiments, B₁₆ cells were injected, via the tail vein into immune-competent C57/BL/6 mice. In this model, tumor colonies in the lung are easily visible within two-three weeks, due to the expression of melanin by the tumor cells. Four days after injection of the B₁₆ cells, treatment with the combination of Adp53, Tf-Adp53 and/or cisplatin (CDDP) was begun. 1×10^{10} viral

pt/mouse/injection (equivalent to 3×10^8 pfu), at a ratio of 1.5×10^5 Tf molecules/virion, was administered systemically via an i.v. tail vein injection three times per week for a total of 12-13 viral treatments. Intraperitoneal CDDP (3-5mg/kg) was administered every 2-4 days with a total of 8-13 doses of CDDP administered. One day after all treatments (five weeks after the initial injection of B₁₆ cells), the lungs were excised from the animals and perfused with 10% formaldehyde.

As shown in Figures 5A-H, there is a dramatic difference in the lungs obtained from the animals receiving the combination treatment and those from the other groups in two separate experiments. While CDDP alone and the Tf-Adp53 alone demonstrated some effect when compared to the lungs from the untreated animals, a significant number of tumor colonies are still evident. Similarly, the lungs of animals treated with the control virus Tf-AdLacZ plus CDDP evidences what is only a drug effect. More significantly, the animals that received the untargeted Adp53 along with CDDP also present with multiple large tumor colonies indicating minimal effect of untargeted Adp53 when systemically delivered. In contrast however, the lungs from the animals that received transferrin-targeted adenoviral p53 (Tf-Adp53) in combination with CDDP are virtually free of obvious tumor metastases. These findings demonstrate that the systemically delivered, Tf-targeted, Adp53 can sensitize tumor cells to conventional chemotherapy in addition to conventional radiotherapy. Moreover, the Tf-Adp53 can also function effectively in a syngeneic mouse model in addition to the nude mouse models used previously.

The Tf molecule employed in all of our experiments is human Tf. It is known that the TfR from a given species can bind the Tf from a range of other species (Aisen, 1998). Nonetheless, we were initially concerned that the selectivity seen with human tumors in nude mice might be attributable to the human Tf we were using being recognized by the tumor TfR in preference to the mouse TfR in the host's normal tissues. The syngeneic model system involving B₁₆ mouse melanoma cells growing in C57/BL/6 mice is reassuring in this regard. In this model, the TfR on the tumor and the TfR on the normal tissues are both mouse TfR. Nonetheless, we were able to demonstrate that adenoviruses complexed with human Tf home to the tumors having elevated levels of mouse TfR. This finding suggests that it is the level of TfR expression rather than a species-related phenomenon that accounts for targeting to the human

xenograft tumors in the nude mouse models described above and boosts the likelihood that we can achieve our ultimate goal of treating human tumors growing in humans.

Example 8

5

Transferrin Targeted Herpes Simplex Virus Transduction

Herpes simplex virus (HSV) is a replication competent viral vector, widely used in gene therapy, especially in the central nervous system (Walker, 1999). As with adenoviral or retroviral vectors, HSV exhibits poor specificity and significant immunogenicity.

10

To explore the feasibility of using the transferrin-targeting strategy to target the replication competent viral vectors, G207, the HSV with a reporter gene LacZ (Walker, 1999), was complexed with human transferrin. G207 (1.1×10^9 pfu/mL, NeuroVir, Inc., in PBS) was mixed with human holo-transferrin (Sigma, 5 mg/mL in water) at different ratios in the same manner as that for the Tf-AdLacZ, as described in Example 1. In one set of experiments, HSV was heat-treated by incubating at 37°C for 10 minutes before mixing with Tf. The heat treatment

15

reportedly can inactivate the HSV.

For transduction experiments, 8×10^4 JSQ-3 cells/well were plated in a 24-well plate. 24 hours later the cells were washed once with EMEM without serum, then 0.3 mL EMEM without serum or antibiotics was added to each well. The HSV or Tf-HSV complexes at different ratios of transferrin to virus in 200 μ L EMEM were added to the wells, at a MOI=1. After 4

20 hours incubation at 37°C, 5% CO₂, with mixing by rotating the test tube once every 2 minutes, 0.5 mL EMEM with 20% serum was added to the wells. After 2 days in culture, the cells were washed once with PBS, and lysed in 1X reporter lysis buffer (Promega). The cell lysates were treated with 100 μ L of 150 μ M 0-nitrophenyl- β -galactopyranoside in 20 mM Tris (pH 7.5) containing 1 mM MgCl₂ and 450 mM β -mercaptoethanol at 37°C for 30 minutes. The reaction

25 was stopped by the addition of 150 μ L/well of 1 M Na₂CO₃. The absorbance was measured at 405 nm. Purified β -galactosidase (Boehringer) was used to make a standard curve. The results were expressed as milliUnits (mU) of β -galactosidase equivalent per mg of total protein. The results are shown in Table 1.

30

Table 1

Transferrin Enhances HSV Transduction Efficiency

	Tf/virion	0	500	1500	5000	15000	50000
	Non-treated*	318	448	463	483	405	675
5	Heat-treated*	286	410	436	455	386	640

* β -galactosidase activity, mU/mg protein

Transferrin enhances the transduction efficiency of HSV, a replication competent viral vector. When HSV was heat-inactivated, the complexing with Tf still showed enhanced transduction efficiency. At a Tf/virion ratio of 50000 and a MOI=1, Tf-HSV gave greater than two-fold more reporter gene expression than did HSV without Tf targeting. The results demonstrate that the transferrin-targeting strategy can be used for replication competent viral vectors such as HSV.

Example 9Transferrin-Targeted Systemic Adenoviral-MediatedGene Delivery *In Vivo* in an MDA-MB-435 Xenograft Nude MouseModel in Conjunction with the Chemotherapeutic Agent Docetaxel (Taxotere)

To further demonstrate the usefulness of this targeted adenoviral delivery system, a second tumor model, the human breast cancer derived cell line MDA-MB-435, was employed. Additionally, since chemotherapy is often the treatment of choice for breast cancer, this experiment tested the ability of the delivery system of this invention to sensitize established human breast cancer xenograft tumors to the commonly used chemotherapeutic agent docetaxel (Taxotere). Xenografts were induced in 4-6 week old female athymic nude (NCR nu-nu) mice by the subcutaneous injection of 2.5×10^6 MDA-MB-435 cells in the mammary fat pad of each animal. Tumors were allowed to develop to a size of 40-50 mm³. The targeted adenovirus, designated Tf Adp53, was prepared by mixing transferrin with adenovirus carrying DNA encoding wt p53 as described in Example 1. The animals were divided into five groups: (i) Untreated (-) Taxotere; (ii) Taxotere alone, (iii) Untargeted Adp53 (+) Taxotere; (iv) Targeted Adp53 (-) Taxotere; (v) Targeted Adp53 (+) Taxotere. The mice were i.v. injected, via the tail vein, every three to four days with 5×10^{10} pfu/mouse/injection of either targeted (an admixture of Tf ligand and virus was injected) or untargeted (no ligand) Adp53. A total of 12 injections

were administered. The day after the initial viral injection, drug treatment was started. The animals were given Taxotere i.v. at a dose of 7.5 mg/kg every three or four days to a total of 11 injections. The tumor sizes were measured weekly in a blinded manner on a total of 6-8 tumors/group. The mean of the tumor volumes per group (mm^3) \pm Standard Error vs. Time (Days) was plotted (Figure 6). While treatment with the Tf-Adp53 alone had no effect, treatment with Taxotere alone or the untargeted Adp53 plus Taxotere induced some growth inhibition indicating a drug effect. However, there was an even more dramatic level of growth inhibition observed in the tumors from the animals receiving Tf-Targeted Ad-p53 in combination with taxotere. These findings show the synergistic effect of the combination treatment and demonstrate not only that the transferrin-targeted Adp53 complex of the invention is effective in multiple human tumor models, but that it can also be used to sensitize tumors to chemotherapeutic agents.

Example 10

Transferrin-Targeted Adenoviral-Mediated Gene

Expression *In Vivo* in a DU145 Xenograft Nude Mouse Model

A prostate cancer derived cell line, DU145, demonstrated improved expression in intratumoral injections. The replication deficient adenovirus serotype 5, that carried the LacZ gene, was used in this example. Athymic nude (nu/nu) mice were subcutaneously injected using Matrigel™ to produce tumors. Tf-Ad-LacZ was prepared as in Example 1. The ratio of Tf/pt was 0.1-0.2 mg/ 10^{10} pt as described in Example 2. The mice had 2 tumors each but only one was injected.

Twenty-four hours after intratumoral injection of 3×10^{10} particles/tumor, the tumors were excised, cut into pieces, flash frozen in liquid nitrogen, and were pulverized in a Bessman tissue pulverizer. β -Galactosidase enzyme activity was measured using the Glacto-Star™ chemiluminescent β -Galactosidase assay system from Tropix, Inc. according to the manufacturer's protocol. The results are shown in Figure 7. Tf-Ad-LacZ gave greater than a 3.4-fold increase in β -galactosidase expression as compared to Ad-LacZ. The results demonstrate that the transferrin-targeting strategy can be used for increasing expression in intratumoral injections.

While the invention has been disclosed in this patent application by reference to the details of preferred embodiments of the invention, it is to be understood that the disclosure is intended in an illustrative rather than in a limiting sense, as it is contemplated that modifications will readily occur to those skilled in the art, within the spirit of the invention and the scope of the
5 appended claims.

LIST OF REFERENCES

- Aisen P (1998). *Met. Ions Biol. Syst.* 35:585-631.
- Asgari K, et al. (1997). *Int. J. Cancer* 71:377-382.
- Baselga J and Mendelsohn J (1994). *Pharmacol. Ther.* 64:127-154.
- Berkner KL (1988). *BioTechniques* 6:616-629.
- Bischoff JR, et al. (1996). *Science* 274:373-376.
- Bristow RG, et al. (1996). *Radiother. Oncol.* 40:197-223.
- Cotten M, et al. (1992). *Proceedings Natl. Acad. Sci. USA* 89:6094-6098.
- Douglas JT, et al. (1996). *Nat. Biotechnol.* 14:1574-1578.
- Goud B, et al. (1988). *Virology* 163:251-254.
- Hall AR, et al. (1998). *Nat. Med.* 4:1068-1072.
- Heise C, et al. (1997). *Nat. Med.* 3:639-645.
- Isaacs WB, et al. (1991). *Cancer Res.* 51:4716-4720.
- Kataoka M, et al. (1998). *Cancer Res.* 58:4761-4765.
- Kirn D, et al. (1998). *Nat. Med.* 4:1341-1342.
- Linke SP (1998). *Nature* 395:13,15.
- Miyamoto T, et al. (1994). *Int. J. Oral. Maxillofac. Surg.* 23:430-433.
- Pirollo KF, et al. (1997). *Oncogene* 14:1735-1746.
- Rogers BE, et al. (1997). *Gene Therapy* 4:1387-1392.
- Roux P, et al. (1989). *Proceedings Natl. Acad. Sci. USA* 86:9079-9083.
- Schwarzenberger P, et al. (1997). *J. Virol.* 71:8563-8571.
- Wagner E, et al. (1992). *Proceedings Natl. Acad. Sci. USA* 89:6099-6103.
- Walker JR, et al. (1999). *Hum. Gene Ther.* 10:2237-2243.
- Weichselbaum RR, et al. (1988). *Int. J. Radiat. Oncol. Biol. Phys.* 15:575-579.
- Wivel NA and Wilson JM (1998). *Hematol. Oncol. Clin. North Am.* 12:483-501.
- Xu L, et al. (1997). *Human Gene Therapy* 8:467-475.
- Xu L, et al. (1999). *Tumor Targeting* 4:92-104.
- U.S. Patent No. 5,108,921
- U.S. Patent No. 5,139,941
- U.S. Patent No. 5,288,641
- U.S. Patent No. 5,378,457

U.S. Patent No. 5,416,016

U.S. Patent No. 5,521,291

U.S. Patent No. 5,547,932

U.S. Patent No. 5,635,382

U.S. Patent No. 5,693,509

U.S. Patent No. 5,762,938

U.S. Patent No. 5,833,975

WO 92/06180

WHAT IS CLAIMED IS:

1. A vector for delivery of a virus to a target cell within a host animal, comprising a cell-targeting ligand non-covalently bound to said virus.
2. The vector of claim 1 wherein said virus and said ligand are not naturally associated with each other.
3. The vector of claim 1 wherein said virus is comprised of a therapeutic nucleic acid.
4. The vector of claim 1 wherein said virus is comprised of a nucleic acid that encodes a therapeutic peptide or protein.
5. The vector of claim 1 wherein said virus is comprised of a nucleic acid that encodes wild-type p53.
6. The vector of claim 1 wherein said virus is a retrovirus or an adenovirus.
7. The vector of claim 1 wherein said virus is selected from the group consisting of adeno-associated virus, herpes simplex virus, cytomegalovirus, vaccinia virus, fowlpoxvirus, canarypoxvirus and Sindbis virus.
8. The vector of claim 1 wherein said virus is a chimeric virus, a hybrid virus, or a recombinant virus.
9. The vector of claim 1 wherein said cell-targeting ligand is selected from the group consisting of proteins, peptides, hormones, antibodies and antibody fragments.
10. The vector of claim 1 wherein said cell-targeting ligand is a native protein or a recombinant protein.

11. The vector of claim 1 wherein said cell-targeting ligand is selected from the group consisting of insulin, toxins, EGF, VEGF, FGF, IGF, heregulin, a viral protein, a bacterial protein, estrogen and progesterone.
12. The vector of claim 1 wherein said cell-targeting ligand is transferrin.
13. The vector of claim 1 wherein said cell-targeting ligand and said virus are present at a ratio in the range of 100 to 1,000,000 ligand molecules per virion.
14. The vector of claim 1 wherein said cell-targeting ligand and said virus are present at a ratio in the range of 6,700 to 400,000 ligand molecules per virion.
15. The vector of claim 1 wherein said cell-targeting ligand and said virus are present at a ratio in the range of 1 μ g to 10 mg of said ligand per 10^{10} virion.
16. The vector of claim 1 wherein said cell-targeting ligand and said virus are present at a ratio in the range of 10 μ g to 600 μ g of said ligand per 10^{10} virion.
17. A method for preparing a vector for the systemic delivery of a virus to a target cell, said vector comprising a cell-targeting ligand non-covalently bound to said virus, comprising mixing said cell-targeting ligand with said virus in an aqueous medium, whereby said ligand non-covalently binds to said virus.
18. The method of claim 17 wherein said aqueous solution includes one or more of a buffering agent, an osmolarity adjusting agent, or an antibiotic.
19. A method for providing a therapeutic agent to an animal in need thereof, comprising administering to said animal a therapeutically effective amount of a vector for delivery of a virus to a target cell within said animal, said vector comprising a cell-targeting ligand non-covalently bound to said virus.

20. The method of claim 19 wherein said animal is a human.
21. The method of claim 19 wherein said therapeutic agent is administered systemically.
22. The method of claim 19 wherein said therapeutic agent is administered parenterally.
23. The method of claim 19 wherein said therapeutic agent is administered intravenously or intra-arterially.
24. The method of claim 19 wherein said therapeutic agent is administered intratumorally.
25. The method of claim 19 wherein said vector encodes wild-type p53.
26. The method of claim 19 wherein said cell-targeting ligand is transferrin.
27. The method of claim 19 wherein said therapeutic agent is administered to an animal receiving chemotherapy in addition to said therapeutic agent.
28. The method of claim 19 wherein said therapeutic agent is administered to an animal receiving radiation treatment in addition to said therapeutic agent.
29. The method of claim 19 wherein said animal has cancer of the head and neck, bladder, breast, thyroid, ovary, brain, prostate, a melanoma or a lymphoma.
30. The method of claim 19 wherein said virus is comprised of a nucleic acid encoding wild-type p53, further wherein said cell-targeting ligand is transferrin, and further whereby said therapeutic agent is administered systemically.
31. The method of claim 30 wherein said therapeutic agent is administered to an animal receiving chemotherapy in addition to said therapeutic agent.
32. The method of claim 30 wherein said therapeutic agent is administered to an animal receiving radiation treatment in addition to said therapeutic agent.

1/7

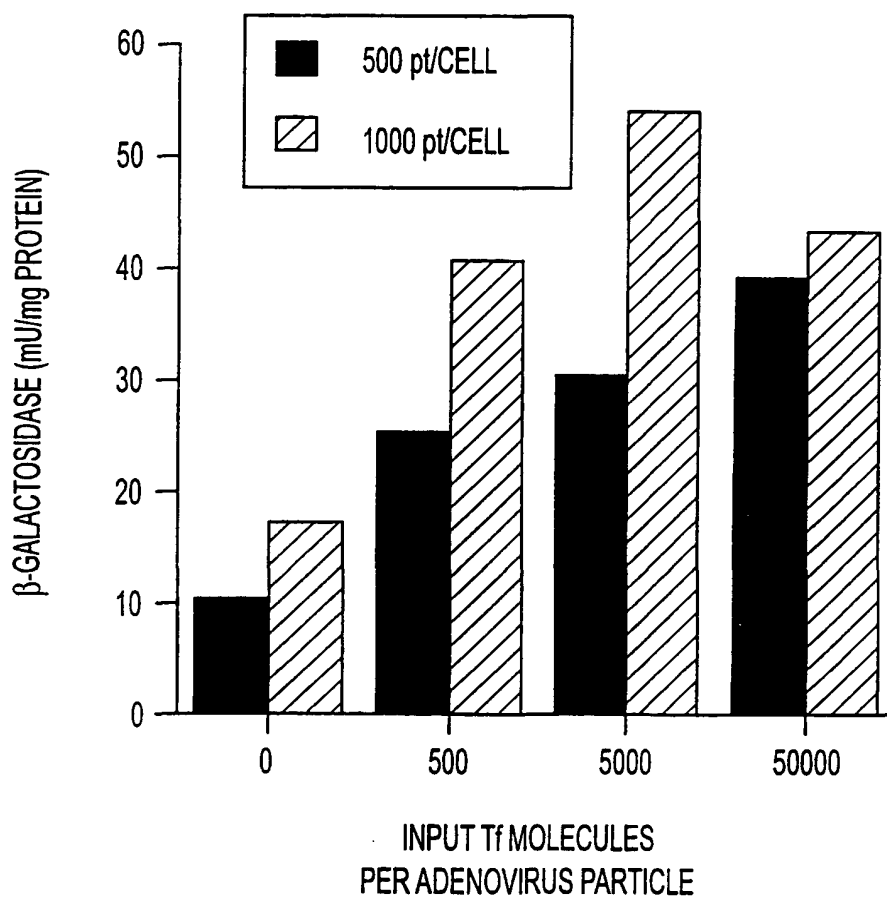


FIG. 1





FIG. 2A

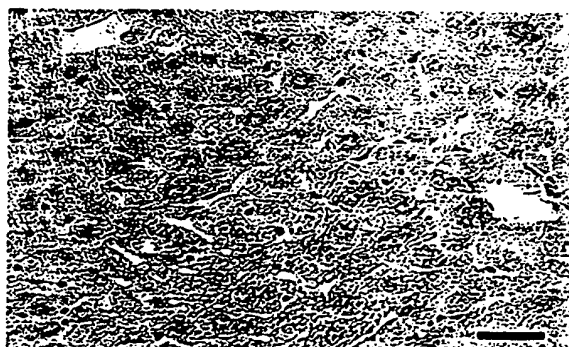


FIG. 2B



FIG. 2C



FIG. 2D



FIG. 2E



FIG. 2F



3/7

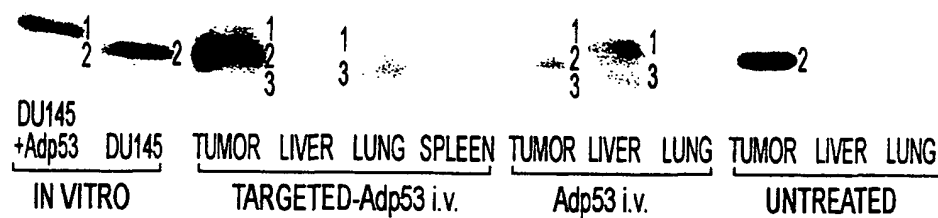


FIG. 3



4/7

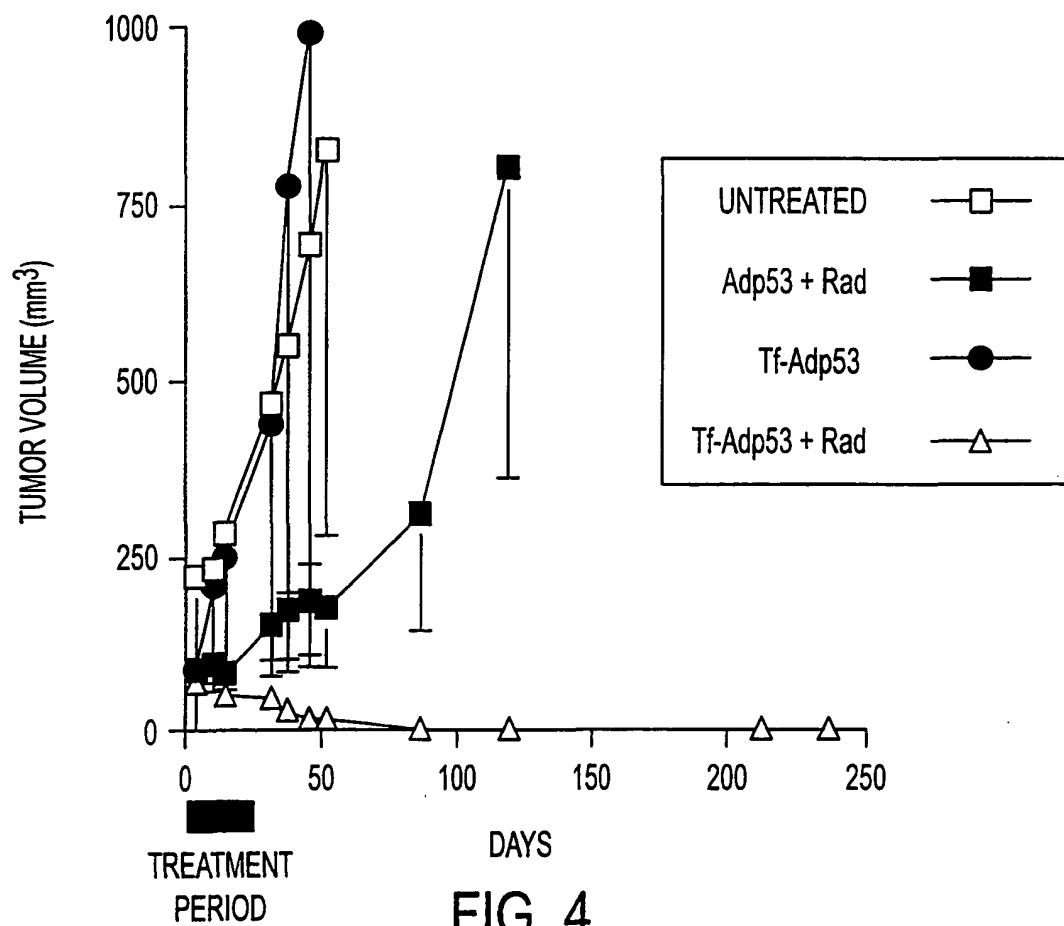


FIG. 4





FIG. 5A



FIG. 5B



FIG. 5C



FIG. 5D



FIG. 5E



FIG. 5F



FIG. 5G



FIG. 5H



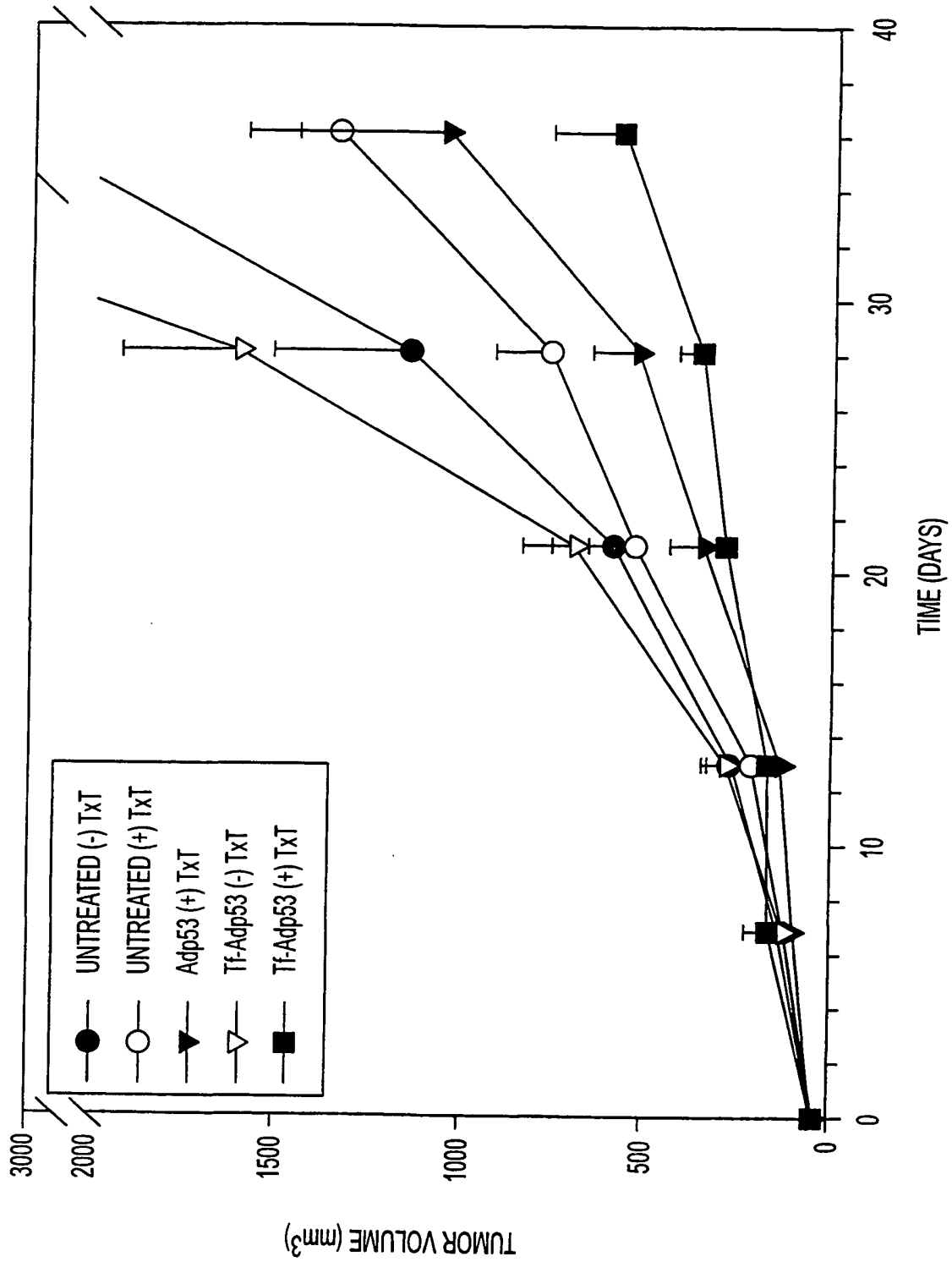


FIG. 6



7/7

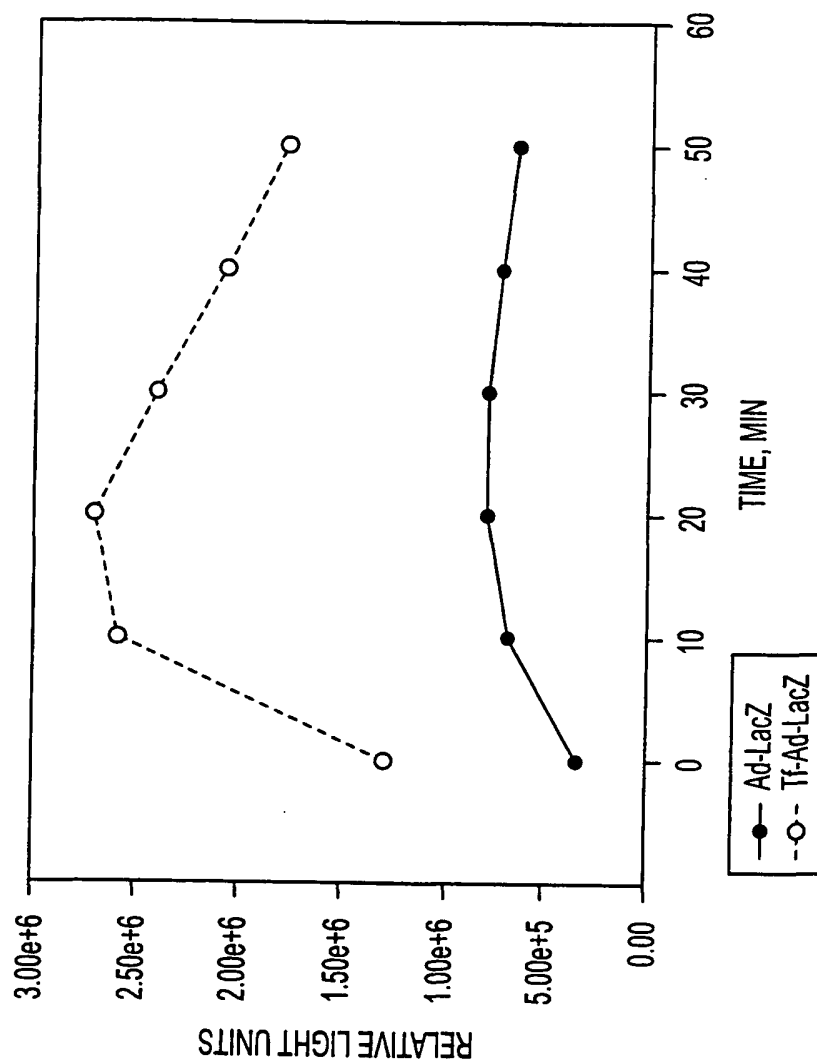


FIG. 7



INTERNATIONALE ANMELDUNG VERÖFFENTLICHT NACH DEM VERTRAG ÜBER DIE
INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT)

(51) Internationale Patentklassifikation ⁵ : C12N 15/87, 15/86, 7/04, A61K 48/00	A1	(11) Internationale Veröffentlichungsnummer: WO 94/24299 (43) Internationales Veröffentlichungsdatum: 27. Oktober 1994 (27.10.94)
--	----	---

(21) Internationales Aktenzeichen: PCT/EP94/01065

(22) Internationales Anmeldedatum: 6. April 1994 (06.04.94)

(30) Prioritätsdaten:
P 43 11 651.5 8. April 1993 (08.04.93) DE(71) Anmelder (für alle Bestimmungsstaaten ausser US):
BOEHRINGER INGELHEIM INTERNATIONAL
GMBH [DE/DE]; Postfach 200, D-55216 Ingelheim am
Rhein (DE). GENENTECH, INC. [US/US]; 460 Point San
Bruno Boulevard, South San Francisco, CA 94080 (US).

(72) Erfinder; und

(75) Erfinder/Anmelder (nur für US): COTTEN, Matthew
[US/AT]; Maxingstrasse 22-24/3/8, A-1130 Wien (AT).
WAGNER, Ernst [AT/AT]; Wiener Strasse 201, A-2103
Langenzersdorf (AT).(74) Anwalt: LAUDIEN, Dieter; Boehringer Ingelheim GmbH, A
Patente, Postfach 200, D-55216 Ingelheim am Rhein (DE).(81) Bestimmungsstaaten: JP, US, europäisches Patent (AT, BE,
CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT,
SE).

Veröffentlicht

Mit internationalem Recherchenbericht.

(54) Title: ADENOVIRUS FOR THE TRANSFER OF FOREIGN DNA INTO HIGHER EUKARYOTIC CELLS

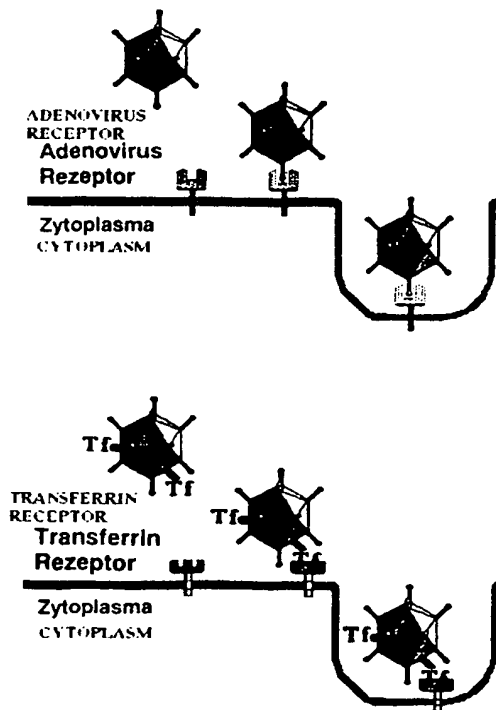
(54) Bezeichnung: ADENOVIRUS FÜR DEN TRANSPORT VON FREMD-DNA IN HÖHERE EUKARIOTISCHE ZELLEN

(57) Abstract

Described is an adenovirus whose surface has been modified with transferrin for the introduction of foreign DNA into higher eucaryotic cells, bonding preferably being through the glycoprotein part of the transferrin. The foreign DNA is imported into cells which have no adenovirus receptors and expressed. The adenovirus may be recombinant or non-recombinant.

(57) Zusammenfassung

Adenovirus für den Transport von Fremd-DNA in höhere eukaryotische Zellen, dessen Oberfläche mit Transferrin modifiziert ist, wobei die Bindung bevorzugt über den Glycoproteinanteil des Transferrins erfolgt. Die Fremd-DNA wird in Zellen, die keine Adenovirusrezeptoren aufweisen, importiert und exprimiert. Das Adenovirus ist rekombinant oder nicht-rekombinant.



LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

AT	Österreich	GA	Gabon	MR	Mauretanien
AU	Australien	GB	Vereinigtes Königreich	MW	Malawi
BB	Barbados	GE	Georgien	NE	Niger
BE	Belgien	GN	Guinea	NL	Niederlande
BF	Burkina Faso	GR	Griechenland	NO	Norwegen
BG	Bulgarien	HU	Ungarn	NZ	Neuseeland
BJ	Benin	IE	Irland	PL	Polen
BR	Brasilien	IT	Italien	PT	Portugal
BY	Belarus	JP	Japan	RO	Rumänien
CA	Kanada	KE	Kenya	RU	Russische Föderation
CF	Zentrale Afrikanische Republik	KG	Kirgisistan	SD	Sudan
CG	Kongo	KP	Demokratische Volksrepublik Korea	SE	Schweden
CH	Schweiz	KR	Republik Korea	SI	Slowenien
CI	Côte d'Ivoire	KZ	Kasachstan	SK	Slowakei
CM	Kamerun	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Tschad
CS	Tschechoslowakei	LU	Luxemburg	TG	Togo
CZ	Tschechische Republik	LV	Lettland	TJ	Tadschikistan
DE	Deutschland	MC	Monaco	TT	Trinidad und Tobago
DK	Dänemark	MD	Republik Moldau	UA	Ukraine
ES	Spanien	MG	Madagaskar	US	Vereinigte Staaten von Amerika
FI	Finnland	ML	Mali	UZ	Usbekistan
FR	Frankreich	MN	Mongolei	VN	Vietnam

ADENOVIRUS FÜR DEN TRANSPORT VON FREMD-DNA IN HÖHERE EUKARIOTISCHE ZELLEN.

Die Erfindung bezieht sich auf das Einbringen von Nukleinsäuren in höhere eukaryotische Zellen.

Bedarf an einem effizienten System für das Einführen von Nukleinsäure in lebende Zellen besteht vor allem im Rahmen der Gentherapie. Dabei werden Gene in Zellen eingeschleust, um *in vivo* die Synthese therapeutisch wirksamer Genprodukte zu erzielen, z.B. um im Falle eines genetischen Defekts das fehlende Gen zu ersetzen.

Für den Transfer von Genen in die Zellen werden z.B. virale Vektoren eingesetzt, die sich der effizienten Eintrittsmechanismen ihrer Ausgangsviren bedienen. Darunter werden Viren verstanden, in deren Genom das in der Zelle zu exprimierende Gen mittels rekombinanter Methoden integriert wurde. Diese Strategie wurde bei der Konstruktion rekombinanter retroviraler und adenoviraler Vektoren angewendet, um einen hoch wirksamen Gentransfer *in vitro* und *in vivo* zu erzielen. Die bisher am weitesten fortgeschrittenen Technologien für die Anwendung von Nukleinsäuren im Rahmen der Gentherapie benutzen retrovirale Systeme für den Transfer von Genen in die Zelle (Wilson et al., 1990; Kasid et al., 1990). Es wurden daher bereits Methoden entwickelt, um die Anwendbarkeit der retroviralen Systeme zu erweitern bzw. deren Spezifität für eine definierte Zellpopulation zu ermöglichen, indem z.B. der Tropismus der Viren verändert wurde.

Von Roux et al., 1989, und in der Französischen Patentanmeldung 2 649 119 wurde ein System beschrieben, das den Tropismus von Retroviren mit Hilfe bifunktioneller Konjugate verändert, die auf der einen

2

Seite einen Antikörper gegen die Virushülle und auf der anderen Seite einen spezifischen Zellmembranmarker für die Zielzellen enthalten und somit die Verbindung des Virus mit der Wirtszelle herstellen.

Der von Goud et al., 1988, vorgeschlagene Ansatz beruht ebenfalls auf dem Prinzip bifunktioneller Konjugate. Diese Konjugate sind eine Konstruktion aus zwei monoklonalen Antikörpern, von denen einer gegen den humanen Transferrinrezeptor und einer gegen das gp70 Hüllprotein des Moloney-Retrovirus gerichtet ist. Mit Hilfe dieser Konjugate konnte das Retrovirus zwar in die Zielzellen eindringen, nicht jedoch in diesen replizieren.

Die in der WO 92/06180 beschriebene Methode zur Veränderung des Tropismus eines Virus besteht darin, die Oberfläche eines Virus mit einem Molekül zu versehen, das an einen Oberflächenrezeptor der Zielzelle bindet, womit das Virus eine - natürlicherweise nicht vorhandene - Spezifität für die Zielzelle erhält. In der WO 92/06180 wird die Modifikation eines Retrovirus und von Hepatitisvirus B mit Kohlenhydratmolekülen beschrieben, die an den Asialoglykoproteinrezeptor binden.

Für die Anwendung im Rahmen der Gentherapie sind in jüngster Zeit an die Stelle rekombinanter Retroviren zunehmend rekombinante Adenoviren getreten (Berkner, 1988; Stratford-Perricaudet et al., 1990; Rosenfeld et al., 1991; Rosenfeld et al., 1992; Stratford-Perricaudet et al., 1992).

Adenovirale Vektoren haben die vorteilhafte Fähigkeit, in nicht-teilende Zellen eindringen und eine DNA-Fremdsequenz im Ausmaß von bis zu 8.5 kb aufnehmen zu

können. Ferner können die Adenoviruspartikel ausgiebig gereinigt werden, ohne an Stabilität einzubüßen, und in Titern höher als 10^{11} PFUs/ml hergestellt werden.

Ein Beschränkung bei der Anwendung der von den Adenoviren Ad2 und Ad5 abgeleiteten rekombinanten Vektoren besteht in ihrer nur geringen Fähigkeit, in Blutzellen einzudringen. Blutzellen sind jedoch ein bevorzugtes Ziel für gentherapeutische Anwendungen, weil sie leicht verfügbar sind und in den Patienten wiedereingeführt werden können, auch sind die Methoden zu ihrer Gewinnung und Kultivierung etabliert. Der Grund für die schwache Aktivität der adenoviralen Vektoren in Blutzellen liegt in der offensichtlich geringen Zahl an Rezeptoren für Adenoviren auf diesen Zellen (Horvath und Weber 1988; Silver und Anderson, 1988). Während die Bindung des Virus an diese Zellen um das Zwei- bis Fünffache reduziert ist, ist die Internalisierung des gebundenen Virus noch wesentlich geringer. Die von vornherein geringe Rezeptorzahl dürfte also mit einer stark verminderten Internalisierung der vorhandenen Rezeptoren einhergehen.

Kürzlich wurde in mehreren Arbeiten der Einsatz von nicht-rekombinanten Adenoviren aufgrund der Fähigkeit von Adenoviren, den Inhalt von Endosomen freisetzen zu können, für den Gentransfer mit DNA-Komplexen mittels Rezeptor-vermittelter Endozytose vorgeschlagen. Der Einsatz von Adenoviren bewirkt eine Steigerung der Effizienz des Gentransfers, indem der Abbau der in die Zelle internalisierten DNA-Komplexe in den Lysosomen vermieden wird (Curiel et al., 1991; Curiel et al., 1992a; Zatloukal et al., 1992; Cotten et al., 1992; Wagner et al., 1992; Curiel et al., 1992b; Yoshimura et al., 1993; WO 93/07283). Es wurde u.a. vorgeschlagen,

die Adenoviren durch Bindung an Polylysin zu modifizieren. Die Adenovirus-Polylysin-Konjugate können zusammen mit Konjugaten aus Transferrin-Polylysin mit DNA komplexiert werden, wobei ternäre Transferrin-Polylysin/Adenovirus-Polylysin/DNA-Komplexe entstehen (Wagner et al., 1992). Im Zuge dieser Arbeit wurde festgestellt, daß K562-Zellen in Gegenwart von freiem Adenovirus bei Transfektion mit Transferrin-Polylysin-Konjugaten nur sehr geringe Expressionsraten des importierten Reportergens zeigten, während Polylysin-gekoppeltes Adenovirus, gemeinsam mit Transferrin-Polylysin unter Bildung eines ternären Komplexes an die Reporter-DNA komplexiert, sehr gute Ergebnisse brachte. Dieses Phänomen ist vermutlich darauf zurückzuführen, daß die Internalisierung der DNA-Komplexe in Blutzellen, die eine geringe Zahl an Adenovirusrezeptoren aufweisen, über den Transferrinrezeptor abläuft, über den die Blutzellen in großer Zahl verfügen.

Adenoviren können nicht in Zellen eindringen, die Adenovirusrezeptoren nicht oder in für eine effiziente Internalisierung ungenügender Zahl aufweisen, oder auch, was vor allem bei Anwendungen *in vivo* von Bedeutung ist, aufgrund der Tatsache, daß die Bindungsstellen des Virus, z.B. durch einen Antikörper, blockiert sind.

Der vorliegenden Erfindung lag die Aufgabe zugrunde, Adenoviren mit der Fähigkeit bereitzustellen, in Zellen, in die sie normalerweise nicht eindringen können, effizient unter Beibehaltung ihrer Fähigkeit zur Genexpression und/oder ihrer endosomolytischen Eigenschaften eindringen zu können.

Die Erfindung betrifft somit ein Virus für den Transport von Fremd-DNA in höhere eukaryotische Zellen, dessen Oberfläche mit einem Liganden für einen Oberflächenrezeptor der Zielzelle derart modifiziert ist, daß es an die Zelle bindet und derart internalisiert wird, daß die Fremd-DNA in der Zelle exprimiert wird. Das Virus ist dadurch gekennzeichnet, daß es ein Adenovirus und der Ligand Transferrin ist.

Es wurde überraschend festgestellt, daß mittels Transferrin-modifiziertem Adenovirus Fremd-DNA in Blutzellen, die der Aufnahme von nicht-modifiziertem Adenovirus nicht zugänglich sind, internalisiert und effizient zur Expression gebracht wird. Bei der Infektion einer Zelle durch ein Virus läuft eine Reihe komplexer Vorgänge ab, die bei den verschiedenen Viren unterschiedlichen Strategien folgen. Da am produktiven Eintritt eines Virus zahlreiche Ereignisse beteiligt sind, die mit der Bindung des Virus an seinen Rezeptor in Zusammenhang stehen, wobei z.B. eine Konformationsänderung, die das Virus bei der Bindung an seinen Rezeptor erfährt, eine für den Ablauf der Internalisierung und Replikation unabdingbare Voraussetzung sein kann, konnte nicht vorausgesagt werden, ob der Zusammenhang der für die Infektion erforderlichen Ereignisse erhalten bleibt, wenn am Virus Modifikationen vorgenommen wurden.

Der Eintritt von Transferrin-modifiziertem Adenovirus über den Transferrinrezeptor (B) im Vergleich zum Eintritt des nicht-modifizierten Virus (A) über seinen Rezeptor ist schematisch in Fig. 1 dargestellt.

Die erfindungsgemäßen Transferrin-modifizierten Adenoviren können verwendet werden, um *in trans* die Aufnahme von Transferrin-Polylysin/DNA-Komplexen in

Zellen zu verbessern, die Transferrin-Rezeptoren, aber keine oder nicht genügend Adenovirus-Rezeptoren aufweisen. Bei der Anwendung *in trans* wird das modifizierte Adenovirus gemeinsam mit Transferrin-Polylysin/DNA-Komplexen auf die zu transfizierenden Zellen aufgebracht, um zusammen mit den DNA-Komplexen in die Zelle aufgenommen zu werden und aufgrund seiner endosomolytischen Fähigkeit die Freisetzung der DNA-Komplexe aus den Endosomen zu bewirken (Curiel et al. 1991).

Das modifizierte Adenovirus kann auch verwendet werden, um als Bestandteil von ternären oder Kombinationskomplexen (Zatloukal et al., 1992) die Effizienz dieser Komplexe zu steigern. Zu diesem Zweck wird das erfindungsgemäße Transferrin-modifizierte Virus, beispielsweise durch eine Biotin-Streptavidin-Brücke, mit einem Polylysin/DNA-Komplex verbunden, wobei gegebenenfalls auch das Polylysin mit Transferrin konjugiert ist. Durch das an das Adenovirus gekoppelte Transferrin übernimmt in diesem Fall das Virus bei Zellen, die Transferrin-Rezeptoren haben, zusätzlich zu seiner endosomolytischen Funktion die Funktion eines Internalisierungsfaktors für den Kombinationskomplex.

Die erfindungsgemäßen Adenovirus-Konjugate können in sämtlichen Anwendungen, in denen Adenoviren eine Steigerung des Gentransfers bewirken, zum Einsatz kommen (Curiel et al., 1991; Curiel et al., 1992a; Zatloukal et al., 1992; Cotten et al., 1992; Wagner et al., 1992; Curiel et al., 1992b; Yoshimura et al., 1993).

In einer Ausführungsform der Erfindung ist das Adenovirus ein rekombinantes Adenovirus, d.h. ein Adenovirus, das in seinem Genom mittels rekombinanter

Methoden integrierte Fremdsequenzen enthält. Dabei sind vor allem Sequenzen von Interesse, deren Expression in der Zielzelle einen erwünschten biologischen Effekt erzielt, z. B. DNA, die ein defektes Gen ersetzt. In dieser Ausführungsform bietet die vorliegende Erfindung den Vorteil, den limitierten Tropismus, den die ansonsten erfolgreiche Verwendung rekombinanter Adenoviren für die Gentherapie aufweist, zu beseitigen und das System breiter anwendbar zu machen.

Für die erfindungsgemäßen Virus-Konjugate besteht keinerlei Beschränkung hinsichtlich der Adenoviruskomponente, sämtliche Adenoviren, die an ihrer Oberfläche eine zur Bindung an Transferrin fähige Gruppierung aufweisen, sind geeignet, z.B. die von Berkner, 1988; Stratford-Perricaudet et al., 1990; Rosenfeld et al., 1991; Rosenfeld et al., 1992; Stratford-Perricaudet et al., 1992, beschriebenen Adenoviren, die als Vektoren für den Import von DNA in die menschliche Zelle, insbesondere für die gentherapeutische Anwendung, vorgeschlagen wurden, können modifiziert werden, um DNA selektiv *in vivo* oder *ex vivo* in die Zielzelle zu befördern.

Im Hinblick auf die Anwendung im Menschen wird als Transferrin-Komponente insbesondere Humantransferrin verwendet. Transferrin ist ein Eisentransportprotein, das mit hoher Effizienz über Rezeptor-vermittelte Endozytose in die Zelle aufgenommen wird, wodurch es als Transport-Vehikel bereits für verschiedene Anwendungen benutzt wurde, z.B. um in Form verschiedener Konjugate Toxine, niedermolekulare Substanzen oder Gene in die Zelle zu transportieren. Der Vorgang, der bei der Internalisierung von Transferrin durch seinen Rezeptor abläuft, unterscheidet sich von anderen Ligand/Rezeptor-Paarungen u.a. dadurch, daß der

Transferrinrezeptor mit hohem Durchsatz rezykliert wird.

Bezüglich der Fremd-DNA bestehen keinerlei durch die vorliegende Erfindung bedingten Beschränkungen; die DNA kann ein beliebiges Gen sein oder auch ein Plasmid-Konstrukt, das z.B. für inhibierende RNA kodierende Elemente enthält. Gentherapeutisch wirksame Sequenzen sind dem Fachmann bekannt; Beispiele für derzeit als für therapeutisch aussichtsreich angesehene Sequenzen sind u.a. der Übersicht von Anderson, 1992, entnehmbar.

Die Erfindung betrifft in einem weiteren Aspekt ein Verfahren zum Einbringen von Fremd-DNA in menschliche Zellen, die keine oder nur eine geringe Zahl von Adenovirus-Rezeptoren aufweisen oder deren Adenovirus-Rezeptoren ganz oder teilweise blockiert sind, in welchem man die Zellen mit dem erfindungsgemäßen Virus in geeigneter Formulierung ex vivo oder in vivo behandelt.

Insbesondere sind die Zellen Blutzellen.

Die Anforderungen an die Formulierung, in der die erfindungsgemäßen modifizierten Viren verabreicht werden, werden durch die spezielle Anwendung definiert; der Fachmann kann einschlägigen pharmazeutischen Handbüchern (z.B. Remington's Pharmaceutical Sciences, 1980) zahlreiche Träger- und Zusatzstoffe entnehmen, die für die Formulierung verwendet werden, wesentlich ist vor allem, daß die Formulierung die Transport-Funktion des erfindungsgemäßen Virus-Transferrin-Konjugats sowie die Bioverfügbarkeit des in der Zelle exprimierten Proteins nicht beeinträchtigen.

Das Virus kann an Transferrin in für die Kopplung von Peptiden an sich bekannter Weise erfolgen, vorzugsweise erfolgt die Bindung des Adenovirus an Transferrin über die Kohlenhydratseitenketten des Transferrins. Dieser Typ Bindung ist in der DE-A1 41 150 38, auf deren Offenbarung hiermit Bezug genommen wird, für Transferrin-Polylysin-Konjugate beschrieben.

Es wurde überraschend festgestellt, daß sich diese Art der Bindung sehr gut zur Modifikation von Adenoviren mit Transferrin eignet, um Fremd-DNA in Zellen zu importieren, die ansonsten dem Transportvehikel Adenovirus nicht bzw. nicht ausreichend zugänglich sind.

Voraussetzung für die Fähigkeit des Virus, an die Kohlenhydratseitenketten des Transferrins zu koppeln, ist das Vorliegen von Aminogruppen an der Oberfläche des Virus. Ohne auf diese Theorie festgelegt sein zu wollen, dürfte es dabei von Vorteil sein, daß die Kohlenhydratseitenkette des Transferrins einen natürlichen Abstandhalter zwischen Transferrin und dem Virus darstellt. Dadurch dürfte einerseits die Bindungs- und Internalisierungsfähigkeit des Transferrins, andererseits dürfte, was für die Anwendung der erfindungsgemäßen Konjugate als Bestandteil von Kombinationskomplexen von Bedeutung ist, die endosomolytische Aktivität des Adenovirus erhalten bleiben.

Ein bevorzugtes Verfahren zur Herstellung der erfindungsgemäßen Adenovirus-Transferrin-Konjugate, das ebenfalls Gegenstand der vorliegenden Erfindung ist, besteht darin, Transferrin unter schonenden Bedingungen zu einer im Kohlenhydratanteil Aldehydgruppen enthaltenden Form zu oxidieren und das oxidierte

Transferrin unter reduzierenden Bedingungen mit dem Adenovirus zu koppeln.

Für den Oxidationsschritt, bei dem terminale Sialinsäuren der Kohlenhydratketten des Transferrins zur Aldehydform oxidiert werden, wird als Oxidationsmittel bevorzugt Perjodat, insbesondere Natriumperjodat, eingesetzt.

Als Substanzen zur Schaffung reduzierender Bedingungen bei der Kopplung der Aldehydform des Transferrins mit dem Adenovirus sind Reduktionsmittel geeignet, die unter schonenden Bedingungen Schiff'sche Basen selektiv reduzieren. Bevorzugt für den Einsatz als Reduktionsmittel im erfindungsgemäßen Verfahren sind Natriumcyanoborhydrid oder tertiäres Butylaminboran.

Das Verfahren wird bevorzugt bei niedrigen Temperaturen, insbesondere 0°C bis Raumtemperatur, durchgeführt.

Die Mechanismen der einzelnen Reaktionsstufen sind dem Durchschnittsfachmann geläufig; es liegt daher im Rahmen seiner Fähigkeiten, die Bedingungen für die einzelnen Verfahrensschritte den jeweiligen individuellen Bedürfnissen anzupassen. In einzelnen Anwendungsfällen kann es erstrebenswert sein, das Viruspartikel mit einer geringeren Anzahl von Transferrinmolekülen auszustatten als in den im Rahmen der vorliegenden Erfindung durchgeführten Versuchen, um ein optimales Gleichgewicht zwischen Internalisierungs- und endosomolytischer Eigenschaft zu erhalten. Modifizierte Adenoviruspartikel mit unterschiedlichen Anteilen von daran gebundenem Transferrin können erhalten werden, indem das Verhältnis Adenovirus/Reagentien empirisch verändert wird.

Figurenübersicht:

- Fig. 1: Schematische Darstellung des Viruseintritts in die Zelle. A: Nicht-modifiziertes Adenovirus. B: Transferrin-modifiziertes Adenovirus.
- Fig. 2: Quantifizierung des an Adenovirus gebundenen Transferrin auf Nitrozellulosemembranblot
- Fig. 3: Expression von β -Galaktosidase in K562-Zellen nach Transfektion mit rekombinantem Adenovirus. A: Nicht-modifiziertes Adenovirus. B: Transferrin-modifiziertes Adenovirus. C: Kontrolle (nicht-infizierte Zellen).
- Fig. 4: Quantitativer Vergleich mittels Luminometrie des Gentransfers in K562-Zellen

Die Erfindung wird anhand des folgenden Beispiels illustriert:

Beispiel

a) Adenoviruspräparation

5×10^6 293-Zellen (ATCC No. 1573; Graham et al., 1977) wurden in 175 cm² Flaschen mit 60 ml DMEM Medium plus 10 % FCS und 1 % Glutamin sowie einem Antibiotikazusatz (Penicillin, Streptomycin) 3 Tage bei 37°C und 5 % CO₂ gezüchtet, bis ca. 2×10^7 Zellen, die zu ca. 80 - 100 % konfluent waren, erhalten wurden. Danach wurde das Medium entfernt und die Zellen mit Adenovirus Ad.RSV β gal (El-, E3-defektes Adenovirus Typ 5, das das E.coli β -Galaktosidasegen unter Kontrolle des RSV Promotor/Enhancers trägt; Stratford-Perricaudet et al.,

1992) infiziert (ca. 2×10^9 Partikel in 5 ml Medium mit 2 % FCS). Nach ca. 2 Tagen Inkubation bei 37°C waren die Zellen angeschwollen und hatten sich nahezu vollständig vom Boden abgelöst. Daraufhin wurden die Zellen 10 min in einem Sorvall GSA Rotor bei 3.000 rpm zentrifugiert, die Zellpellets mit PBS in ein 50 ml Röhrchen transferiert und 10 min in einer Heraeus Zentrifuge bei 1.000 rpm zentrifugiert. Das Pellet wurde im 2- bis 3fachen Volumen 10 mM HEPES/1 mM EDTA (HE) aufgenommen, in flüssigem Stickstoff schockgefroren und bei -70°C gelagert. Für den Zellaufschluß wurden die Zellen vier Frier-/Tauzyklen (flüssiger Stickstoff/37°C Wasserbad) unterworfen und 10 min zentrifugiert (Heraeus, 4.000 rpm). Anschließend wurde das Zellysats einer Ultrazentrifugation unterworfen (vTI-50 47.000 rpm/1 h/20°C; CsCl Dichtegradient: 20 ml 1.33 g/cm^3 CsCl in HE, unterschichtet mit 10 ml 1.45 g/cm^3 CsCl in HE, überschichtet mit 10 ml Zellysats). Die opaleszente Virusbande wurde gesammelt und einer zweiten Ultrazentrifugation unterworfen (vTI-50 63.000 rpm/4 h/20°C; CsCl Gleichgewichtsgradient: 2.5 ml 1.33 g/cm^3 CsCl in HE, gemischt mit 2.5 ml Virusbande). Pro Zellkulturflasche wurden $1 - 3 \times 10^{11}$ Viruspartikel erhalten, die mit 40 % (v/v) Glycerin bei -70°C gelagert wurden. Die Bestimmung der Virion-Konzentration wurde über den Proteingehalt mittels Bradfordassay (BSA, Fraktion V, BMB als Standard) bestimmt, wobei das Verhältnis 1 mg/ml Virusprotein = 1.34×10^{12} Viruspartikel (Lemay et al., 1980) herangezogen wurde.

b) Modifikation des Adenovirus mit Transferrin

Eine Lösung von 105 mg (1.30 μmol) Transferrin (human, Sigma) in 1 ml 30 mM Natriumacetatpuffer (pH 5) wurde

einer Gelfiltration auf einer Sephadex G-25 PD10 Säule (Pharmacia) unterworfen, wobei derselbe Puffer verwendet wurde. Die erhaltenen 2 ml Lösung, die 80 mg (1.0 μmol) Transferrin enthielten (der Transferringehalt wurde durch UV-Messung bei 280 nm und Ninhydrinassay bestimmt), wurden in einem Speedvac (Savant) auf 1 ml konzentriert, auf 0°C gekühlt und mit 50 μl 30 mM Natriumacetatpuffer (pH 5), enthaltend 1.1 mg (5 μmol) Natriumperjodat, behandelt. Die Mischung wurde in einem Eisbad im Dunklen 90 min lang stehen gelassen. Anschließend wurde eine weitere Gelfiltration (Sephadex G-25 PD10 Säule, Pharmacia, 150 mM NaCl, 10 mM HEPES, pH 7.3) durchgeführt, dabei wurden 1 ml einer Lösung, enthaltend 66 mg (0.82 μmol) oxidiertes Transferrin erhalten (der Gehalt an oxidiertem, Aldehyd enthaltender Form von Transferrin wurde bestimmt mittels Färbung mit Anisaldehydreagens, wie von Wagner et al., 1991, beschrieben). Ein Teil der modifizierten Transferrinlösung (0.6 ml; 0.5 μmol) wurde rasch zu einer Lösung, enthaltend 25 μg (bezogen auf Protein) Adenovirus in 300 μl HBS, hinzugefügt. Nach 1 h bei Raumtemperatur wurde eine Lösung von 1 mg (15 μmol) Natriumcyanborhydrid zugegeben. Nach 24 h bei Raumtemperatur wurde das Transferrin-konjugierte Adenovirus von überschüssigem freiem Transferrin gereinigt, indem das Virus mit einem gleichen Volumen HBS (150 mM NaCl, 20 mM HEPES, pH 7.4) verdünnt und das Material über einen CsCl-Stufengradienten zentrifugiert wurde. Ca. 1.5 ml des modifizierten Virus wurden in einem vTI-65 Röhrchen (Beckman) mit 3 ml 1.31 g/cm³ CsCl und 1 ml 1.45 g/cm³ CsCl (beide CsCl-Lösungen in 1 mM EDTA, 20 mM HEPES, pH 7.4) unterschichtet. Die Probe wurde 1 h lang bei 63.000 rpm in einem vTi-65 Rotor (Beckman) zentrifugiert. Die opaleszente Virusbande wurde geerntet, mit einem gleichen Volumen HBS verdünnt und einer zweiten identischen CsCl-

Gradientenreinigung unterworfen. Alternativ wurde die erste Virusbande mit 1.33 g/cm^3 CsCl, 20 mM HEPES, 1 mM EDTA, pH 7.4, auf 5.5 ml gebracht und die Probe bis zum Gleichgewicht zentrifugiert (4 h, 63.000 rpm, vTi-65 Rotor). Das gereinigte modifizierte Virus wurde geerntet, mit einem gleichen Volumen von 96 % Glyzerin (Fluka) verdünnt und bis zur Verwendung bei -70°C gelagert. Parallel wurde eine Kopplung mit eisenfreiem Transferrin durchgeführt; das gereinigte Virus wurde mit 1 μl 1 mM Eisen (III) Citratpuffer, pH 7.5 versetzt.

c) Quantitative Bestimmung des an Adenovirus gebundenen Transferrins

Serienverdünnungen von Transferrin-modifiziertem Adenovirus, nicht-modifiziertem Adenovirus sowie Transferrin-Standards (jeweils in HBS; die Anzahl der aufgetragenen Viruspartikel bzw. Transferrinmoleküle ist Fig. 2 entnehmbar) wurden an eine Nitrozellulosemembran (Schleicher & Schuell, 0.1 mm Porengröße) gebunden. Der Blot wurde über Nacht bei 4°C mit 3 % (w/v) pulverisierter Magermilch in HBS (10 ml) vorhybridisiert. Der Transferringehalt jeder Probe wurde bestimmt, indem die Membran 4 h bei Raumtemperatur einem Mausantikörper, der humanes Transferrin erkennt (Chemicon MAB 033-19/1, Verdünnung 1:2000 in HBS/Milch, 10 ml), und im Anschluß daran (nach 2 stündigem Waschen mit 4 x 130 ml HBS/Milch) 1 h lang einem ^{125}I -markiertem zweiten Antikörper (Schaf-anti-Maus-Ig, Amersham, Kat.No. L52215, 2 μCi , in 20 ml HBS/Milch) exponiert wurde. Die Phosphoimager-Analyse der Membran zeigt die Gegenwart von Transferrin in den Proben mit modifiziertem Adenovirus, jedoch kein Signal beim nicht-modifizierten Adenovirus. Der Vergleich der mit den modifizierten Virus erhaltenen Signale mit den

Transferrinstandards erlaubt eine Bestimmung der Menge an Transferrin, die an ein Virusteilchen gebunden ist. Es wurde gefunden, daß 3×10^9 Viruspartikel ein Signal geben, das mit dem von 4×10^{12} Transferrinmolekülen vergleichbar ist. Daraus ergibt sich, daß an ein Adenovirusteilchen ca. 1.000 Transferrinmoleküle gebunden sind. Die Assoziation der Transferrinmoleküle mit Adenovirusteilchen durch zwei CsCl-Dichtegradienten spricht für eine kovalente Bindung zwischen Kapsidproteinen des Virus und dem Transferrinmolekül. Damit in Einklang ist auch das Ergebnis einer Analyse von Viruskapsidproteinen mittels SDS-PAGE, die zeigte, daß die Hauptmenge des gekoppelten Transferrins an das Hexon gebunden ist.

- d) Transfektion von K562-Zellen mit Transferrin-modifiziertem rekombinantem Adenovirus, enthaltend als Fremd-DNA das β -Galaktosidasegen

K562-Zellen (ATCC No. CCL 243) wurden in Suspension in RPMI 1640 Medium (Gibco BRL) plus 10 % FCS, 100 Einheiten/ml Penicillin, 100 $\mu\text{g/ml}$ Streptomycin und 2 mM Glutamin gezüchtet. 20 h vor der Transfektion wurden die Zellen in frisches Medium, enthaltend zwecks Erhöhung ihrer Transferrinrezeptor-Zahl 50 μM Deferrioxamin (Cotten et al., 1990), überführt. Die Transfektionen wurden bei einer Dichte von 250.000 Zellen/ml in demselben Medium (plus 50 μM Deferrioxamin) durchgeführt. Es wurden jeweils gleiche Mengen (3.000 Viruspartikel/Zelle bis 300 Viruspartikel/Zelle) von entweder nicht-modifiziertem Adenovirus (Ad.RSV β gal) oder von modifiziertem Adenovirus (Tf-Ad.RSV β gal) auf die K562-Zellen aufgebracht. 48 h nach der Transfektion wurden ca. 25.000 Zellen in einem Volumen von 100 bis 200 μl in die Vertiefungen einer rundbödigen 96-Well-Platte

überführt und bei 800 rpm in einem Beckman GH 3.7 Rotor 10 min lang zentrifugiert. Der Überstand wurde vorsichtig entfernt und mit 100 µl 0.5 % Glutardialdehyd in HBS ersetzt. Die Zellen wurden durch Pipettieren dispergiert und dann wieder zentrifugiert. Daraufhin wurde das Fixiermittel entfernt und die Zellen gewaschen. Anschließend wurde mit 200 µl Färbelösung (10 mM Phosphatpuffer pH 7.0, 150 mM NaCl, 1 mM MgCl₂, 3.3 mM K₄Fe(CN)₆·3H₂O, 3.3 mM K₃Fe(CN)₆ und 0.2 % 5-Brom-4-chlor-3-indolyl-β-galaktopyranosid) bei 37 °C 20 min bis 3 h inkubiert (Lim und Chae, 1989). Die Zellen wurden bei 38°C 5 h lang inkubiert, dann 2 x gewaschen und nach dem Transfer in flachbödige Vertiefungen einer 96-Well-Platte photographiert.

Das Ergebnis der Transfektionen ist in Fig. 3 wiedergegeben: Es wurde gefunden, daß beim höchsten Virus/Zellverhältnis das nicht-modifizierte Virus die Zellen mit einer Leistungsfähigkeit von ca. 5 % transduzieren kann. Beim selben Virus/Zellverhältnis transduziert jedoch das Transferrin-modifizierte Virus mehr als 90 % der Zellpopulation, wobei auch die tatsächlichen Mengen an pro Zelle produzierter β-Galaktosidase höher sind. Kontrollversuche zeigten, daß sowohl nicht-modifiziertes als auch Transferrin-modifiziertes Adenovirus in HeLa-Zellen mit gleicher Leistungsfähigkeit eindringen konnte.

Zusätzlich wurde eine Analyse der β-Galaktosidase-Aktivität mittels Luminometrie nach der von Jain und Magratz, 1991, beschriebenen Methode durchgeführt. Dazu wurden K562-Zellen 18 h lang in RPMI/10 % FCS, enthaltend 50 µM Deferrioxamin gezüchtet. Unmittelbar vor der Infektion wurden die Zellen in frisches Deferrioxamin enthaltendes Medium gegeben (250.000 Zellen/ml, 50.000 Zellen pro Vertiefung einer Platte

mit 96 Vertiefungen). Die Verdünnungen der Kontrollen und der Proben mit Transferrin-modifiziertem Adenovirus Ad.RSVßgal wurden in RPMI/2 % hitzeinaktiviertem Pferdeserum hergestellt und Aliquots des Virus (50 µl), die die in der Fig. 4 angegebenen Anzahl an Viruspartikeln pro Zelle enthielten, auf die Zellen aufgegeben. Nach 4 h bei 37°C wurden die Zellen in frischem Medium (ohne Deferrioxamin) gewaschen. Nach 24 h bei 37°C wurden Aliquots von 50.000 infizierten oder Kontrollzellen zur luminometrischen Messung mittels Zentrifugation gesammelt, in 100 µl 0.25 M Tris pH 7.5 aufgenommen und durch drei Frier/Auftauzyklen (flüssiger Stickstoff/37°C) aufgebrochen. Die Zelltrümmer wurden durch Zentrifugieren entfernt (14.000 x g, Eppendorf), und Aliquots des Überstandes, auf Proteingehalt standardisiert, wurden unter Verwendung des chemilumineszierenden Substrats AMPGD und einem Emerald Lumineszenzverstärker (Tropix) analysiert. Das Ergebnis der Messungen ist in Fig. 4 dargestellt.

Literatur

- Anderson, F.W. , 1992, Science 256, 808.
- Berkner, K.L., 1988, BioTechniques 6, 616-629.
- Cotten, M., Wagner, E., Zatloukal, K., Phillips, S.,
Curiel, D.T. und Birnstiel, M.L., 1992,
Proc.Natl.Acad.Sci. USA 89, 6094-6098.
- Cotten, M., Laengle-Rouault, F., Kirlappos., H.,
Wagner, E., Mechtler, K., Zenke, M., Beug, H., und
Birnstiel, M.L., 1990, Proc.Natl.Acad.Sci. USA 87,
4033-4037.
- Curiel, D.T., Agarwal, S., Wagner, E. und Cotten, M.,
1991, Proc.Natl.Acad.Sci. USA 88, 8850-8854.
- Curiel, D.T., Agarwal, S., Romer, M.U., Wagner, E.,
Cotten, M., Birnstiel, M.L. und Boucher, R.C.,
1992a, Am.J.Respir.Cell and Mol.Biol. 6, 247-252.
- Curiel, D.T., Wagner, E., Cotten, M., Birnstiel, M.L.,
Agarwal, S., Li, Ch.-M., Loechel, S. und Hu, P.-
H., 1992b, Human Gene Therapie 3, 147-154.
- Goud, B., Legrain, P. und Buttin, G., 1988, Virology
163, 251-254.
- Graham, F., Smiley, J., Russel, W.C. und Nairu, R.,
1977, J. Gen. Virol. 36, 59-72.
- Horvath, J. und Weber, J., 1988, J. Virol. 62, 341-345.
- Jain, V.K. und Magrath, I.T., 1992, Anal. Biochem. 199,
119-124.
- Kasid, A., Morecki, S., Aebersold, P., Cornetta, K.,
Culver, K., Freeman, S., Director, E., Lotze,
M.T., Blaese, R.M., Anderson, W.F. und Rosenberg,
S.A., 1990, Proc.Natl.Acad.Sci. USA 87, 473-477.
- Lemay, P., Boudin, M., Milleville, M. und Boulanger,
P., 1980, Virology 101, 131-143.
- Lim, K. und Chae, C.B., 1989, BioTechniques 7, 576-579.
- Rosenfeld, M.A., Siegfried, W., Yoshimura, K.,
Yoneyama, K., Fukayama, M., Stier, L.E., Paakko,
P.K., Gilardi, P., Stratford-Perricaudet, L.D.,

- Perricaudet, M. et al., 1991, Science 252, 431-434.
- Rosenfeld, M.A., Yoshimura, K., Trapnell, B., Yoneyama, K., Rosenthal, E., Dalemans, W., Fukayama, M., Bargon, J., Stier, L., Stratford-Perricaudet, L.D. et al., 1992, Cell 68, 143-155.
- Roux, P., Jeanteur, P. und Piechaczyk, M., 1989, Proc.Natl.Acad.Sci. USA 86, 9079-9083.
- Stratford-Perricaudet, L., Levrero, M., Chasse, J.-F., Perricaudet, M. und Briand, P., 1990, Hum. Gene Ther. 1, 241-256.
- Stratford-Perricaudet, L., Makeh, I., Perricaudet, M. und Briand, P., 1992, J. Clin. Invest. 90, 626-630.
- Wagner, E., Cotten, M., Mechtler, K., Kirlappos, H. und Birnstiel, M.L., 1991, Bioconjugate Chemistry 2, 226-231.
- Wagner, E., Zatloukal, K., Cotten, M., Kirlappos, H., Mechtler, K., Curiel, D.T. und Birnstiel, M.L., 1992, Proc.Natl.Acad.Sci. USA 89, 6099-6103.
- Wilson, J.M., Danos, O., Grossman, M., Raulet, D.H. und Mulligan, R.C., 1990, Proc.Natl.Acad.Sci. USA 87, 439-443.
- Yoshimura, K., Rosenfeld, M.A., Seth, P. und Crystal, R.G., 1993, J. Biol. Chem. 268, 2300-2303.
- Zatloukal, K., Wagner, E., Cotten, M., Phillips, S., Plank, C., Steinlein, P., Curiel, D. und Birnstiel, M.L., 1992, Ann.New York Acad.Sci. 660, 136-153.
- Remington's Pharmaceutical Sciences, 1980, Mack Publ. Co., Easton, PA, Osol (ed.).

Patentansprüche

1. Virus für den Transport von Fremd-DNA in höhere eukaryotische Zellen, dessen Oberfläche mit einem Liganden für einen Oberflächenrezeptor der Zielzelle derart modifiziert ist, daß es an die Zelle bindet und derart internalisiert wird, daß die Fremd-DNA in der Zelle exprimiert wird, dadurch gekennzeichnet, daß das Virus ein Adenovirus und der Ligand Transferrin ist.
2. Modifiziertes Adenovirus nach Anspruch 1, dadurch gekennzeichnet, daß das Virus und das Transferrin über die Kohlenhydratseitenketten des Transferrins miteinander verbunden sind.
3. Modifiziertes Virus nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß das Virus ein Adenovirus vom Typ 2 ist.
4. Modifiziertes Virus nach Anspruch 1 oder 2, dadurch gekennzeichnet, daß das Virus ein Adenovirus vom Typ 5 ist.
5. Modifiziertes Virus nach einem der vorhergehenden Ansprüche, dadurch gekennzeichnet, daß das Virus ein rekombinantes Adenovirus ist, das als Fremd-DNA eine gentherapeutisch wirksame DNA-Sequenz enthält.
6. Modifiziertes Virus nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, daß das Transferrin Humantransferrin ist.
7. Verfahren zur Herstellung von Adenovirus-Transferrin-Konjugaten nach Anspruch 1, dadurch gekennzeichnet, daß man Transferrin unter

schonenden Bedingungen zu einer im Kohlenhydratanteil Aldehydgruppen enthaltenden Form oxidiert und das oxidierte Transferrin unter reduzierenden Bedingungen mit dem Adenovirus koppelt.

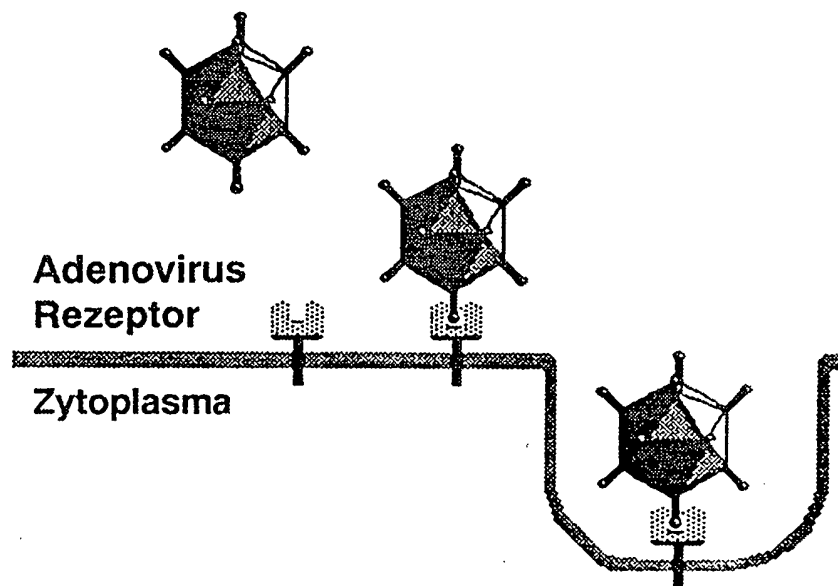
8. Verfahren nach Anspruch 7, dadurch gekennzeichnet, daß man das Transferrin mit Periodat oxidiert.
9. Verfahren nach Anspruch 7 oder 8, dadurch gekennzeichnet, daß man die Kopplung unter reduzierenden Bedingungen in Gegenwart einer Substanz durchführt, die unter für das Transferrin schonenden Bedingungen Schiff'sche Basen selektiv reduziert.
10. Verfahren nach Anspruch 9, dadurch gekennzeichnet, daß man die Kopplung in Gegenwart von Natriumcyanoborhydrid vornimmt.
11. Verfahren zum Einführen von Fremd-DNA mittels humanem Adenovirus in humane Zellen, die keine oder nur eine geringe Zahl von Adenovirus-Rezeptoren aufweisen oder deren Adenovirus-Rezeptoren ganz oder teilweise blockiert sind, dadurch gekennzeichnet, daß man die Zellen mit einem in den Ansprüchen 5 und 6 definierten Virus in Berührung bringt.
12. Verfahren zum Einführen von Fremd-DNA mittels humanem Adenovirus in humane Zellen, die keine oder nur eine geringe Zahl von Adenovirus-Rezeptoren aufweisen oder deren Adenovirus-Rezeptoren ganz oder teilweise blockiert sind, dadurch gekennzeichnet, daß man die Zellen mit einem in einem der Ansprüche 1 bis 4 und 6

definierten Virus als Bestandteil eines Komplexes in Berührung bringt, in welchem Polylysin mit dem Virus sowie gegebenenfalls zusätzlich mit Transferrin konjugiert sowie mit der Fremd-DNA komplexiert ist.

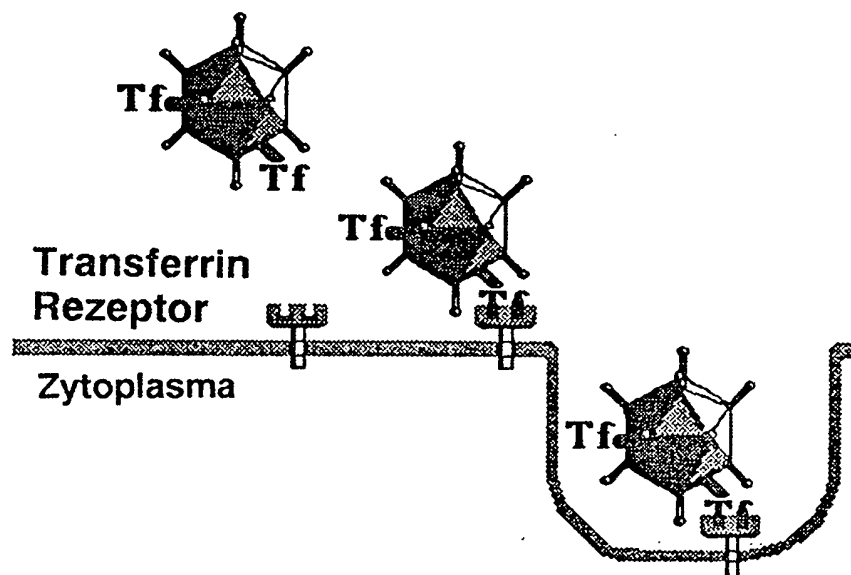
13. Verfahren nach Anspruch 11 oder 12, dadurch gekennzeichnet, daß die Zellen Blutzellen sind.

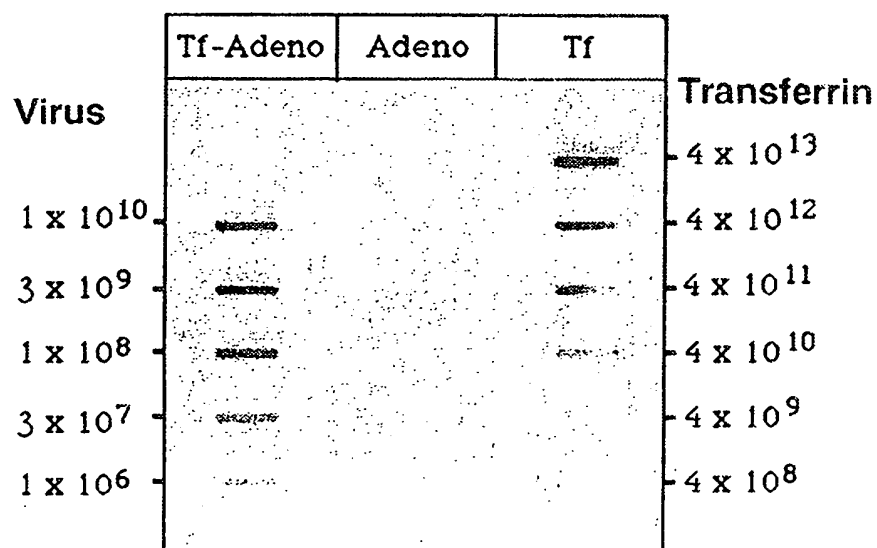
1/4
Fig. 1

A.

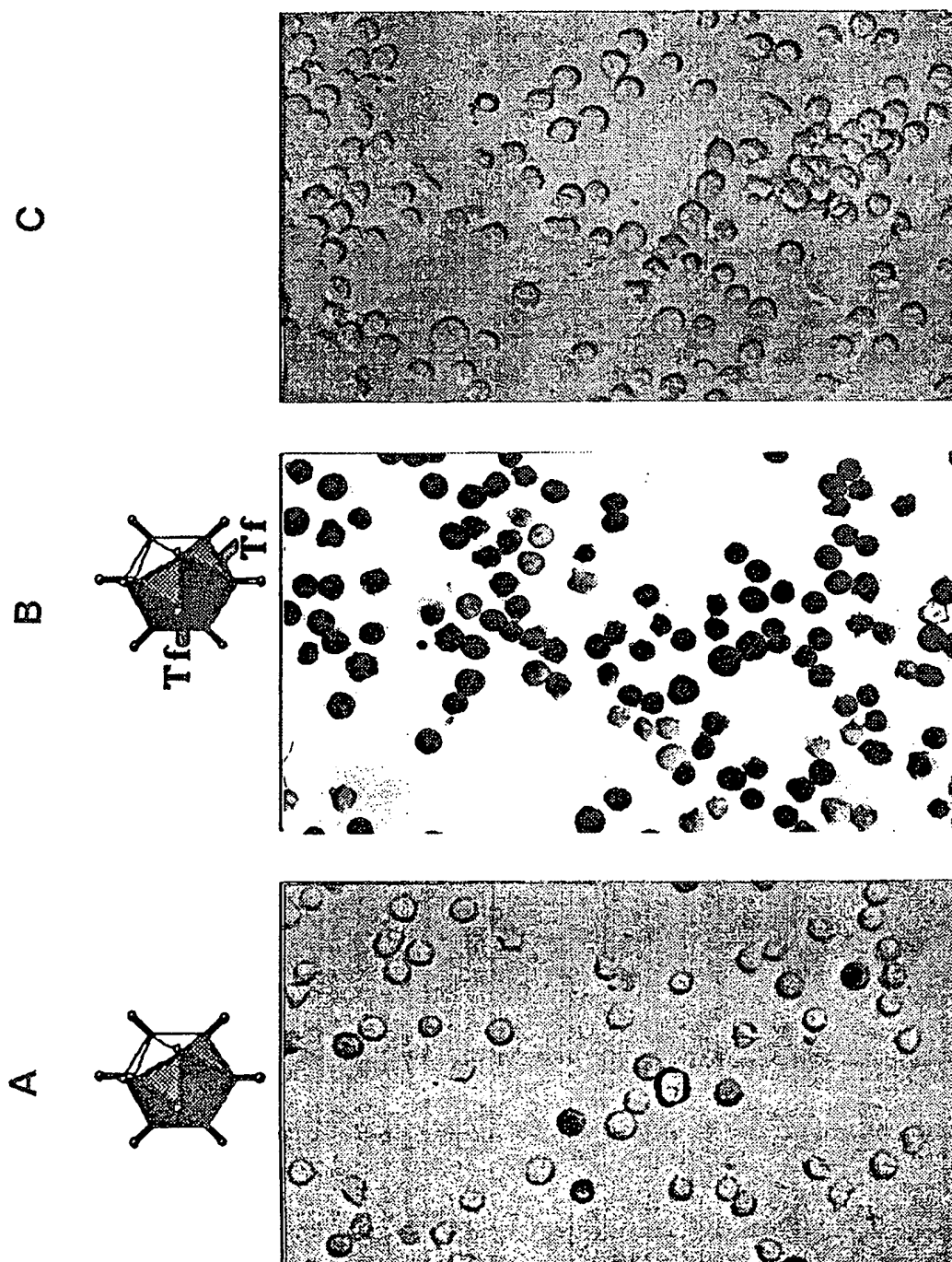


B.



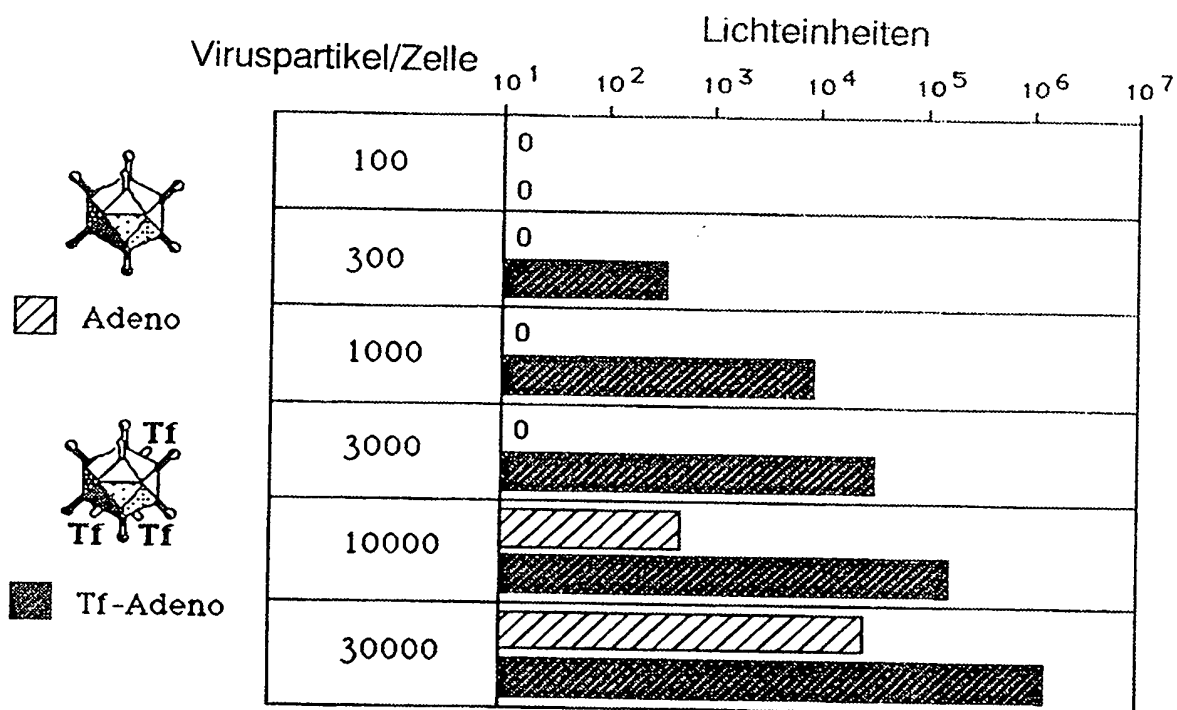
2/4
Fig. 2

3/4
Fig. 3



4/4

Fig. 4



A. CLASSIFICATION OF SUBJECT MATTER
 IPC 5 C12N15/87 C12N15/86 C12N7/04 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
E	WO,A,94 10323 (IMPERIAL CANCER RESEARCH TECHNOLOGY LTD) 11 May 1994 see page 10, line 16 - line 17; claims ---	1,4,5,11
Y	WO,A,92 06180 (UNIVERSITY OF CONNECTICUT) 16 April 1992 see the whole document --- -/--	1-6, 11-13

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

26 July 1994

Date of mailing of the international search report

- 4. 08. 94

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
 Fax (+ 31-70) 340-3016

Authorized officer

Chambonnet, F

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 89, no. 13 , July 1992 , WASHINGTON US pages 6099 - 6103 WAGNER, E. ET AL. 'Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes' see the whole document ---	1-6, 11-13
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 88, no. 19 , October 1991 , WASHINGTON US pages 8850 - 8854 CURIEL, D.T. ET AL. 'Adenovirus enhancement of transferrin-polylysine-mediated gene delivery' cited in the application see the whole document ---	1-6, 11-13
P,Y	WO,A,93 07283 (BOEHRINGER INGELHEIM INTERNATIONAL GMBH & UNIVERSITY OF NORTH CAROLINA) 15 April 1993 see the whole document ---	1-6, 11-13
P,Y	WO,A,93 09221 (THERAGENE HB) 13 May 1993 see the whole document ---	1-6, 11-13
P,Y	WO,A,94 06923 (UNIVERSITY OF CONNECTICUT) 31 March 1994 see the whole document -----	1-6, 11-13

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Bd. 89, Nr. 13, Juli 1992, WASHINGTON US Seiten 6099 - 6103 WAGNER, E. ET AL. 'Coupling of adenovirus to transferrin-polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes' siehe das ganze Dokument ---</p>	1-6, 11-13
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. Bd. 88, Nr. 19, Oktober 1991, WASHINGTON US Seiten 8850 - 8854 CURIEL, D.T. ET AL. 'Adenovirus enhancement of transferrin-polylysine-mediated gene delivery' in der Anmeldung erwähnt siehe das ganze Dokument ---</p>	1-6, 11-13
P,Y	<p>WO,A,93 07283 (BOEHRINGER INGELHEIM INTERNATIONAL GMBH & UNIVERSITY OF NORTH CAROLINA) 15. April 1993 siehe das ganze Dokument ---</p>	1-6, 11-13
P,Y	<p>WO,A,93 09221 (THERAGENE HB) 13. Mai 1993 siehe das ganze Dokument ---</p>	1-6, 11-13
P,Y	<p>WO,A,94 06923 (UNIVERSITY OF CONNECTICUT) 31. März 1994 siehe das ganze Dokument -----</p>	1-6, 11-13

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
WO-A-9410323	11-05-94	KEINE	
WO-A-9206180	16-04-92	AU-A- 8860391	28-04-92
		CA-A- 2092323	02-04-92
		EP-A- 0553235	04-08-93
WO-A-9307283	15-04-93	AU-A- 2652692	03-05-93
		CA-A- 2118816	31-03-93
		EP-A- 0545016	09-06-93
		FI-A- 941474	30-03-94
WO-A-9309221	13-05-93	SE-A- 9103183	01-05-93
WO-A-9406923	31-03-94	KEINE	

Formblatt PCT/ISA/210 (Anhang Patentfamilie)(Juli 1992)

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 94/01065

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9410323	11-05-94	NONE	
WO-A-9206180	16-04-92	AU-A- 8860391 CA-A- 2092323 EP-A- 0553235	28-04-92 02-04-92 04-08-93
WO-A-9307283	15-04-93	AU-A- 2652692 CA-A- 2118816 EP-A- 0545016 FI-A- 941474	03-05-93 31-03-93 09-06-93 30-03-94
WO-A-9309221	13-05-93	SE-A- 9103183	01-05-93
WO-A-9406923	31-03-94	NONE	

Form PCT/ISA/210 (patent family annex) (July 1992)

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 5 C12N15/87 C12N15/86 C12N7/04 A61K48/00

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 5 C12N A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
E	WO,A,94 10323 (IMPERIAL CANCER RESEARCH TECHNOLOGY LTD) 11. Mai 1994 siehe Seite 10, Zeile 16 - Zeile 17; Ansprüche	1,4,5,11
Y	WO,A,92 06180 (UNIVERSITY OF CONNECTICUT) 16. April 1992 siehe das ganze Dokument -/--	1-6, 11-13

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

☒ Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :

- * "A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
- * "E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
- * "L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
- * "O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
- * "P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

* "T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

* "X" Veröffentlichung von besonderer Bedeutung, die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderscher Tätigkeit beruhend betrachtet werden

* "Y" Veröffentlichung von besonderer Bedeutung, die beanspruchte Erfindung kann nicht als auf erfinderscher Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

* "&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

26. Juli 1994

Absendedatum des internationalen Recherchenberichts

- 4. 08. 94

Name und Postanschrift der Internationale Recherchenbehörde
Europäisches Patentamt, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+ 31-70) 340-3016

Bevollmächtigter Bediensteter

Chambonnet, F